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DIAGNOSIS AND MANAGEMENT OF TRICHOTHECENE TOXICOSIS IN THE SWINE MODEL

ANNUAL AND FINAL REPORT

AD-A205 208

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September 1, 1988

SUPPORTED BY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5224

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20030204140

89 3 07 043

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: unclassified

1b. RESTRICTIVE MARKINGS:

2a. SECURITY CLASSIFICATION AUTHORITY:

2b. DECLASSIFICATION / DOWNGRADING SCHEDULE:

3. DISTRIBUTION / AVAILABILITY OF REPORT: approved for public release; distribution unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S):

5. MONITORING ORGANIZATION REPORT NUMBER(S):

6a. NAME OF PERFORMING ORGANIZATION: University of Illinois

6b. OFFICE SYMBOL (If applicable):

7a. NAME OF MONITORING ORGANIZATION:

6c. ADDRESS (City, State, and ZIP Code): Department of Veterinary Biosciences
College of Veterinary Medicine
University of Illinois, Urbana, IL 61801

7b. ADDRESS (City, State, and ZIP Code):

8a. NAME OF FUNDING / SPONSORING ORGANIZATION: U.S. Army Medical Research and Development Command

8b. OFFICE SYMBOL (If applicable):

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-85-C-5224

8c. ADDRESS (City, State, and ZIP Code): Fort Detrick
Frederick, Maryland 21701-5012

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
63763A	3M2-63763D807	AK	033

11. TITLE (Include Security Classification): Diagnosis and Management of Trichothecene Toxicosis in the Swine Model

12. PERSONAL AUTHOR(S): W. Buck, W. Haschek-Hock, R. Lambert, S. Swanson, V. Beasley, M. Biehl, M. Busse, K. Coddington, A. Dahlem, F. Galey, C. Helaszek, C. Keferlis, B. Kindler, C. Knupp

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
annual*/final	FROM 7-1-85 TO 1-31-88	1988 September 1	463

16. SUPPLEMENTARY NOTATION: *annual report covers the period 1 October 1986 to 31 January 1988

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	11	
06	04	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): RAI
see attached

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

see attached

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION: unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Virginia Miller	(301) 663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

12. N. Little, G. Lundeen, R. Manuel, R. Mariner, R. McCartney, V. Pang, R. Pfeiffer, R. Poppenga, H. Rood, T. Sakamoto, C. Schaeffer, D. Schaeffer, R. Wong-Pack

18. Subject Terms

T-2 toxin, therapy, cutaneous therapy, combination therapy, drug therapy, metabolism, GI blood flow, superactivated charcoal, pathophysiology, histopathology, ascorbic acid, dexamethasone, dexamethasone pharmacokinetics, methylprednisolone, Ambersorb resin, diacetoxyscirpenol, deoxynivalenol, biotransformation, deepoxidation, analysis, hydrolysis, hydroxylation, conjugation, mass spectra, metabolism, (KT)

19. Abstract

Therapy: The efficacy of a variety of approaches for the treatment of iv induced acute T-2 toxicosis was assessed in rats and swine. 1) Oral (po) superactivated charcoal (SAC), intravenous (iv), dexamethasone (DEX), intraperitoneal (ip), methylprednisolone (MPSS), but not ip ascorbic acid, were effective post-toxin therapeutic agents in rats as measured by increases in survival times (ST). 2) Pre- and post-toxin (iv) treatment of rats with po SAC resulted in increased ST and decreased lesion severity in the duodenum, jejunum, and ileum. 3) SAC or Ambersorb resin XE-348F was effective in adsorbing T-2 toxin *in vitro*, and in prolonging the STs of rats given lethal (8 mg/kg) po doses of the toxin followed by po SAC or resin at 1 g/kg or less. SAC was a more effective binding agent than the resin. 4) Similarities in pharmacokinetic data for DEX in rats indicated that they may be an acceptable model for humans. The plasma half-life of DEX in rats was prolonged as a result of T-2 toxicosis. 5) Oral SAC or po SAC and cathartics were effective in reducing clinical signs and lesions in swine given lethal po doses of T-2 toxin. 6) At all time points, SAC or a soap and water wash (SOAP) were effective in reducing the severity of skin lesions induced by prior topical application of the toxin to swine. The combination of SAC and SOAP was most effective and SOAP alone was more effective than SAC alone. 7) Combinations of po SAC and magnesium sulfate, iv metoclopramide, iv DEX, iv sodium bicarbonate and normal saline effectively improved STs of swine given highly lethal iv doses of T-2 toxin. 8) Swine given 0.6 or 2.4 mg/kg T-2 toxin iv had reduced gastric and small intestinal blood flow at all time points, while there was an increase in large intestinal blood flow by 3 hr and a decrease by 6 hr. 9) Assessment of DEX and SAC combination therapy for rats given lethal doses (0.8 to 1.2 mg/kg) of T-2 toxin indicated that: predosing with DEX was more effective at increasing ST than giving DEX immediately or post-toxin exposure; predosing with SAC increased ST; increasing the time between SAC doses decreased ST; when the first dose of SAC was given after DEX, ST was decreased; when given in the proper order, the combination of DEX and SAC was of greater benefit than either treatment alone.

Detection and Fate: The role of fecal and intestinal microflora on the metabolism of trichothecene mycotoxins was examined in this study. Suspensions of fecal microflora obtained from horses, cattle, dogs, rats, swine, and chickens were incubated anaerobically with the trichothecene mycotoxin, diacetoxyscirpenol (DAS). Microorganisms from rats, cattle, and swine completely biotransformed DAS, primarily

to the deacylated deepoxidation products, DE MAS and DE SCP. By contrast, fecal microflora from chickens, horses, and dogs failed to reduce the epoxide group. Intestinal microflora obtained from rats completely biotransformed DAS to DE MAS, DE SCP, and SCP, and T-2 toxin to the deepoxy products, deepoxy HT-2 (DE HT-2) and deepoxy T-2 triol (DE TRIOL). Rat intestinal microflora also biotransformed the polar trichothecenes, T-2 tetraol and scirpenetriol, to their corresponding deepoxy analogs. Deepoxy T-2 toxin (DE T-2) was synthesized from T-2 toxin and demonstrated to be 400 times less toxic than T-2 toxin in the rat skin irritation bioassay and non-toxic to mice at doses of 60 mg/kg intraperitoneally. Since deepoxidation is a significant detoxification reaction for trichothecenes, variation in intestinal microflora among animals may account, at least in part, for the species variability in toxicity.

Biotransformation of the trichothecene mycotoxin T-2 by the hepatic S-9 fraction prepared from phenobarbital-treated rats yielded a new metabolic product identified as 4'-hydroxy T-2. The metabolite 4'-hydroxy T-2 was shown to be deacylated at the C-4 position to yield 4'-hydroxy HT-2 when incubated with rat hepatic S-9 preparations.

Metabolic profiles of the excreta from rats following i.v., oral, and dermal administration of tritium-labeled T-2 toxin at 0.15 mg/kg and 0.60 mg/kg were determined. The major metabolites in urine were 3'OH HT-2, T-2 tetraol, 3'OH HT-2, and unknown metabolites M5, M7, and M9. The metabolite labeled M9 (major metabolite) was tentatively identified as deepoxy 3'OH HT-2. There was no significant effect on metabolic profiles due to dose, but there was a variable effect associated with the route of administration. The increase over time of appreciable levels of deepoxy metabolites as a percentage of extracted radioactivity was both consistent and statistically significant.

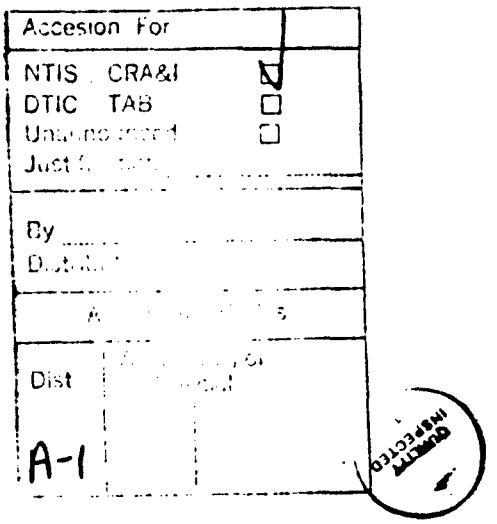
The enterohepatic circulation of T-2 toxin and its conjugated metabolites was examined in bile-cannulated male rats. The thin-layer radiochromatographic metabolite profiles of the bile following intravenous and intraduodenal administration of T-2 toxin were similar. No free metabolites of T-2 toxin were detected in the bile of any animals administered the purified conjugates. Oral treatment of the rats with the b-glucuronidase inhibitor saccharic acid lactone did not produce a statistically significant decline in the amount of radioactivity recovered in the bile following administration of the glucuronide conjugates.

Metabolic and elimination profiles were investigated in rabbits following intravenous, dermal and oral administration of tritium-labeled T-2 toxin at 0.15 and 0.6 mg/kg body weight. The major metabolites detected in the urine were T-2 tetraol (TOL), deepoxy T-2 tetraol (DE TOL), 3'OH HT-2 and an unknown metabolite labeled M9. In feces, the major products eliminated were HT-2, 3'OH HT-2, DE TOL, and M9.

A gas chromatographic method for screening trichothecene mycotoxins in feeds was developed. Deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), T-2 toxin, and their fungal metabolites are hydrolyzed to their corresponding parent alcohols (DON, NIV, scirpenetriol, or T-2 tetraol) by alkaline hydrolysis. After derivatization, the toxins are quantitated by gas chromatography with electron capture

detection. Confirmation and increased sensitivity can be achieved by negative chemical ionization mass spectrometry with no additional sample workup.

Two types of antibodies against T-2 toxin, namely anti-T-2-HB-BSA and anti-3-Ac-NEOS-HS-BSA, showed good cross-reactivity with deepoxy T-2 toxin. Our results indicate that the epoxide is not an important epitope for the production of antibody against T-2 toxin.



SUMMARY

I. DEVELOPMENT OF PREVENTATIVE AND THERAPEUTIC REGIMENS FOR T-2 TOXICOSIS

The efficacy of a variety of approaches for the treatment of intravenously (iv) induced acute T-2 toxicosis was assessed utilizing young female rats. A single large dose (30 mg/kg, ip) of the water-soluble salt of methylprednisolone given 15 min after the toxin significantly prolonged survival times in iv T-2 toxin (1 mg/kg) treated animals. Other agents given ip did not prolong survival times at the dosages employed. These included diltiazem hydrochloride, dazemgrel, N-acetylcysteine, dimethyl sulfoxide, adenosine triphosphate (ATP), ATP combined with magnesium chloride (ATP-$MgCl_2$), ascorbic acid, and aprotinin (all given after the toxin) and trichodermin given either before or after the toxin. Trichodermin, a trichothecene mycotoxin like T-2, appeared to have a detrimental effect on survival time whether given 1 hr prior to or 1 hr after T-2 toxin.

Studies were undertaken to assess the therapeutic efficacy of 3 superactivated charcoal oral dosing protocols for the treatment of acute parenterally induced T-2 toxicosis in rats. One gram superactivated charcoal (dry weight) per kg body weight given as a suspension in normal saline via gavage either immediately, 4, and 8 hr after the administration of T-2 toxin intravenously (iv) at 0.6 mg/kg, or immediately and 6 hr after the administration of T-2 toxin iv at 0.75 mg/kg, was not effective in improving survival rates over control groups given T-2 toxin iv (at either 0.6 or 0.75 mg/kg) followed by oral gavage with normal saline (0.9%). However, rats given 1 gram of superactivated charcoal per kg at 13 and 1 hr prior to and 6 hr after the administration of T-2 toxin iv at 0.8 mg/kg had significantly improved survival rates when compared to a control group given the toxin and normal saline via gavage at an identical dosing regimen as the treated rats. It appears that in rats, pretreatment with superactivated charcoal is an effective therapeutic approach for the treatment of acute T-2 toxicosis induced by iv exposure to the toxin.

A semiquantitative scoring system was used to assess changes in the severity of histologic tissue lesions in rats given methylprednisolone sodium succinate (MPSS) ip at 30 mg/kg 15 min after the administration of T-2 toxin iv at 1.0 mg/kg. Control animals given T-2 toxin but no therapy developed histologic lesions characteristic of acute T-2 toxicosis in lymphoid tissues, the gastrointestinal tract, pancreas, and adrenals. There was a significant decrease in the severity of tissue lesions in the glandular portion of the stomach and the spleen in the group treated with MPSS compared to the positve control group. In a group given MPSS alone, lymphocyte necrosis was noted in the cortex of the thymus. Therapy had no effect on the severity of tissue lesions in the duodenum, jejunum, ileum, cecum, pancreas, or adrenals.

A semiquantitative scoring system was used to assess changes in the severity of histologic tissue lesions in rats given superactivated charcoal (SAC) po at 1 gram/kg 13 hr and 1 hr before and 6 hr after the administration of T-2 toxin iv at 0.8 mg/kg (an approximate LD_{50} dose). Control rats given T-2 toxin but no SAC developed histologic lesions characteristic of acute T-2 toxicosis in lymphoid tissues such as the thymus, spleen, mesenteric lymph nodes and Peyer's patches, gastrointestinal tract, pancreas, and adrenal

glands. In rats given T-2 toxin + SAC, there was a marginally significant decrease in the severity of tissue lesions in the duodenum and jejunum ($p = 0.054$ and 0.052, respectively) and a significant decrease in lesion severity in the ileum and closely associated lymphoid tissue (Peyer's patches) ($p = 0.025$ and 0.033, respectively). Therapy had no effect on the severity of lesions in the thymus, spleen, mesenteric lymph nodes, stomach, cecum, pancreas, and adrenal gland.

Ascorbic acid (AA) was given ip to female Sprague Dawley rats 12 hr prior to and immediately after (group 1) or immediately after (group 2) the iv administration of a dose of T-2 toxin (1 mg/kg) approximately 1.5X the LD_{50} to assess its efficacy as a treatment for acute T-2 toxicosis. In addition, the slow iv infusion of prostaglandin E_1 (PGE_1) over a 2 hr period at 50 µg/kg or a combination therapy, consisting of an iv bolus of dexamethasone sodium phosphate (DEX) at 6 mg/kg and a slow iv infusion of PGE_1 at 5, 25, or 50 µg/kg, was evaluated as an immediate post-T-2 toxin treatment in the rat. Ascorbic acid did not improve survival at any dose evaluated and, in fact, had a detrimental effect on median survival times when given ip either immediately after or 12 hr before and immediately after the administration of T-2 toxin. PGE_1 given alone at 50 µg/kg by slow iv infusion did not improve survival. The combination of DEX and PGE_1 did improve survival times compared with the control group of animals given T-2 toxin and no therapy. However, survival with the combination therapy was no better and, in the case of the groups given PGE_1 at either 25 or 50 µg/kg, was apparently worse than that obtained with DEX alone.

Three groups of swine (6 per group) were used to study blood flow alterations associated with T-2 toxin-induced shock. Low and high dose groups were given T-2 toxin iv at 0.6 or 2.4 mg/kg, respectively; the third group served as a vehicle (70% ethanol) control. Radiolabeled microspheres were administered into the left atrium to assess organ blood flow before dosing and at 90-minute intervals until 6 hours postdosing.

Gastric blood flow was reduced postdosing in both T-2 toxin-dosed groups, but the reduction was more severe at the high dose. At 6 hours, the gastric blood flow of the high dose group had declined to 17% of this group's predose value. The maximal reduction in gastric blood flow of the low dose group, to 30% of the group's predose value, was observed at 3 hours postdosing; by 6 hours, it had increased to 49%.

Small intestinal blood flow of the control group gradually declined over the 6 hours to 64% of the predose value. The high dose group displayed a maximal increase in small intestinal blood flow at 3 hours to 174% of the predose value. This was followed by a reduction at 6 hours to 62% of the predose value, at which time the swine were experiencing a severe decline in cardiac output to 37% of the predose value. Blood flow to the small intestine of the low dose group was increased approximately 60% at 1.5 hours and then gradually decreased to the control value over the remainder of the observation period.

The large intestinal blood flow of the high dose group increased at 3 hours to 177% of the predose value and declined, but only very late in the experiment, as indicated by a mean of 100% of the predose value at 4.5 hours followed by 66% at 6 hours. The large intestinal blood flow of the low dose

group, however, was increased to 151, 196, 200, and 142% of the predose value for this group at 1.5, 3, 4.5, and 6 hours, respectively.

The reduction in gastric blood flow is likely to be related to the frequent development, in swine given high doses of T-2 toxin, of a grossly bright red gastric fundus with histologic evidence of vascular congestion in the submucosa and mucosal degeneration and necrosis. The increased blood flow in the intestine may account for the lesser susceptibility of these areas to the acute effects of the toxin.

Oral superactivated charcoal (1 g/kg) or superactivated charcoal and cathartics (sorbitol, magnesium sulfate, or sodium sulfate, also at a dose of 1 g/kg) when given within 2 minutes of a lethal oral dose of 10 mg/kg T-2 toxin were of benefit in alleviating clinical signs of T-2 toxicosis in swine.

There were no apparent detrimental effects caused by the administration of a cathartic with superactivated charcoal, which was in contrast with results obtained in the rat model. Additionally, gross lesions associated with oral exposure to T-2 toxin were minimal or absent in swine treated with superactivated charcoal or superactivated charcoal and sorbitol.

T-2 toxin (6 mg) dissolved in 90% DMSO was topically applied to nine 9-cm^2 sites (1.67 mg T-2/cm^2) on the dorsum of each of 9 young, crossbred, SPF, female pigs, 20.6 $\pm$ 1.9 kg in weight. A superactive charcoal paste (SAC) or a soap and water wash (SOAP) or a combination of these 2 treatments was applied to 8 of the T-2 exposed sites on each animal. These treatments were applied at various times postexposure ranging from 5 to 65 minutes. The site which received T-2 alone served as a positive control. DMSO was applied to a tenth site on each pig as a negative control. Animals were killed 1, 3, or 6 days after treatment. Skin lesions were examined and graded grossly and histologically. No adverse systemic clinical signs were observed in any of the animals. Marked reddening and slight swelling of the T-2 toxin treated positive control sites was present throughout the study. Ulceration of this site was first noted on Day 3. All therapeutic regimens effectively reduced lesion severity resulting from T-2 toxin application. Significant differences in relative effectiveness were also seen between treatments. In each significant pair, the ordering of mean lesion severity was SAC + SOAP < SAC or SOAP alone and SOAP < SAC. As a single treatment, SOAP appears to be more effective than SAC in reducing lesion severity. These results failed to provide unequivocal evidence of an additive therapeutic effect when SAC and SOAP were used sequentially on the same site.

T-2 toxin was administered intravenously to swine at a dose of 3.6 mg/kg body weight (iv LD_{50} approximately 1.2 mg/kg). Four different therapeutic protocols were assessed for their efficacy in the treatment of the resultant acute T-2 toxicosis syndrome. One therapeutic protocol included the combined use of metoclopramide (1 mg/kg iv immediately prior to toxin and 0.25 and 1.25 hr after the toxin), oral (po) activated charcoal (1 g/kg), magnesium sulfate (1 g/kg, po), dexamethasone sodium phosphate (6 mg/kg, iv, multiple doses after the toxin), sodium bicarbonate (variable amounts, iv), and normal saline (all therapy). The other 3 protocols utilized the same agents less 1 of the following: sodium bicarbonate, normal saline, or the combination of activated charcoal and magnesium sulfate. All 4 treatment

groups had improved survival times compared to a positive T-2 control group. Within the limits of the study, it would appear that the removal of activated charcoal and magnesium sulfate was most detrimental to the T-2 toxin dosed swine.

Four therapeutic protocols utilizing different combinations of dexamethasone sodium phosphate (DEX, multiple doses of 6 mg given iv, after the toxin), normal saline (SAL, iv), sodium bicarbonate (BICARB, variable amounts, iv), and superactivated charcoal (SAC, 2 g/kg, po) + magnesium sulfate (MS, 1/2 g/kg) were evaluated for oral efficacy in swine given an acutely lethal dose (3.6 mg/kg) of T-2 toxin iv. A number of physiologic parameters known to be affected by T-2 toxin were measured including hemodynamic, blood-gas, hematologic, and clinical chemistry variables. There were no clearcut effects of therapy on hemodynamic variables. The iv administration of normal saline did not maintain aortic mean pressure but did appear to result in a relative degree of hemodilution, an increase in urine production, and amelioration of elevations in serum concentrations of potassium, phosphorus, and creatinine. The iv administration of sodium bicarbonate lessened the decline in arterial blood pH. SAC, given orally, improved survival but did not appear to have a significant effect on measured parameters. The oral administration of MS caused an increase in serum magnesium concentrations. The antiemetic, metoclopramide, as given to all swine in the study, did not prevent emesis induced by T-2 toxin.

Both SAC and Ambersorb resin XE-348F removed greater than 99% of the added T-2 toxin within the 15-minute in vitro incubation period at adsorbent: toxicant ratios of 20:1 or greater. At lower ratios, however, SAC was the superior adsorbent, removing an average of 97% of the T-2 at a ratio of 2.5:1, compared to an average of only 17% bound by the resin at the same ratio.

Oral ambersorb resin was an effective therapy (as measured by survival times and rates) when given at 1 g/kg body weight as a 10% solution immediately after an oral dose of 8 mg/kg T-2 toxin. Althougn the resin and a similar oral dose of superactivated charcoal were equally effective when given immediately, when given after a lapse of 1 or 3 hours, the charcoal was of significantly greater benefit. This may be a reflection of the slightly greater binding capacity of the charcoal, as shown in the in vitro experiments. Calculating from the effective dose curves, the ED_{50} values and 95% fiducial limits were 0.40 g/kg (0.28, 0.52) for the resin and 0.09 g/kg (0.003, 0.18) for the superactivated charcoal.

The pharmacokinetics of dexamethasone in rats was investigated. The kinetic parameters following im and iv administration of dexamethasone at 10 mg/kg were statistically indistinguishable. The plasma half-life of dexamethasone was 2.6 h with a total body clearance of 0.266 l/kg/h and a volume of distribution of 1.1 l/kg after either iv or im administration. There was some suggestion of dose-dependent pharmacokinetics. The results obtained by reverse phase high performance liquid chromatography (RP-HPLC) were similar in magnitude and variability to published results obtained by radioimmunoassay (RIA). Allometric relationships were developed for clearance, CL (l/h) = 0.255 $W^{1.004}$ using our results and published pharmacokinetic and body mass, W (kg), data for humans, cattle, horses, and dogs. These data show that the rat may be an acceptable model for the study of human

therapeutic dosage regimens with dexamethasone because of the similarity in its pharmacokinetic parameters with those of humans.

The disposition of plasma dexamethasone was determined in female Sprague-Dawley rats given T-2 toxin. Dexamethasone was given intravenously (IV) or intramuscularly (IM) immediately or 1 h after rats had been given an approximate LD_{50} dose of T-2 toxin (0.75 mg/kg, IV). Plasma concentrations were compared to that of control animals dosed with dexamethasone alone. Rats given dexamethasone IV immediately or 1 h after exposure to T-2 toxin had plasma concentrations which were similar and significantly higher than controls after 2.5 h. Animals treated immediately IM had significantly ($p < 0.05$) higher plasma concentrations than controls after 8 h, while those treated after a 1 h delay had higher plasma concentrations than controls by 4 h. In contrast, plasma dexamethasone concentrations in animals given dexamethasone IM immediately after the toxin differed from those given the delayed therapy by 1 h.

Dexamethasone (10 mg/kg) was given intramuscularly (IM) as a single dose, or in a multiple dosage regimen, beginning 1 h after intravenous (IV) T-2 toxin administration (1 mg/kg). Subsequent doses (from 1 to 7) of dexamethasone and/or saline were given at 5 h intervals thereafter. Multiple dosing had no beneficial effect. Neither median survival time nor mortality differed significantly from the T-2 toxin dosed controls. However, the median survival time decreased as the number of doses of dexamethasone increased.

Swine were treated IV with several different therapeutic agents either alone or in combinations following administration of 2.4 to 3.6 mg/kg T-2 toxin IV. Phenoxybenzamine, a nonspecific α-blocker, administered (1 mg/kg) by IV drip, appeared to improve peripheral perfusion and delay the onset of diarrhea in 2 swine compared to a positive T-2 control animal. Propranolol, a ß-blocker, hastened the decline in cardiac output and decreased the time to death in 2 swine given the drug at 0.15 mg/kg, as compared to a control animal. Survival time was enhanced in 2 pigs receiving either dexamethasone (8, 4, and 2 mg/kg) or methylprednisolone sodium succinate (15.2 mg/kg twice) along with bicarbonate and fluid therapy.

Experiments were conducted to define the optimum times and doses of dexamethasone (DEX) im and the optimum times of administration of oral superactivated charcoal (SAC, 1 g/kg) for rats given a lethal iv dose of T-2 toxin (0.8 to 1.2 mg/kg). Statistical analysis of the survival data using response surface analysis and stepwise regression yielded quadratic polynomial models which indicated the following: 1) predosing with DEX was more effective at increasing survival time than was giving DEX immediately or post T-2 exposure; 2) predosing with SAC increased survival time; 3) increasing the time between SAC doses decreased survival time; 4) when the first dose of SAC was given after the DEX, survival was decreased; and 5) when given in the proper order, the combination of DEX and SAC was of greater benefit than either treatment alone. Treatment combinations were identified which resulted in corrected survival times of 155 hr after an iv dose of toxin (1.2 mg/kg) which gave median survival times of 13 hr in sham-treatmed positive controls.

II. FATE AND DETECTION OF T-2 TOXIN AND ITS METABOLITES AS INFLUENCED BY DOSAGE ROUTE OF ADMINISTRATION, SPECIES, AND THERAPEUTIC INTERVENTION

The role of fecal and intestinal microflora on the metabolism of trichothecene mycotoxins was examined in this study. Suspensions of microflora obtained from the feces of horses, cattle, dogs, rats, swine, and chickens were incubated anaerobically with the trichothecene mycotoxin, diacetoxyscirpenol (DAS). Microorganisms from rats, cattle, and swine completely biotransformed DAS, primarily to the deacylated deepoxidation products, deepoxy monoacetoxyscirpenol (DE MAS) and deepoxy scirpentriol (DE SCP). By contrast, fecal microflora from chickens, horses, and dogs failed to reduce the epoxide group in monoacetoxyscirpenol (MAS) and scirpentriol (SCP), in addition to unmetabolized parent compound. Intestinal microflora obtained from rats completely biotransformed DAS to DE MAS, DE SCP, and SCP; and T-2 toxin to the deepoxy products, deepoxy HT-2 (DE HT-2) and deepoxy T-2 triol (DE TRIOL). Rat intestinal microflora also biotransformed the polar trichothecenes, T-2 tetraol and scirpentriol, to their corresponding deepoxy analogs. Deepoxy T-2 toxin (DE T-2) was synthesized from T-2 toxin and demonstrated to be 400 times less toxic than T-2 toxin in the rat skin irritation bioassay and nontoxic to mice given 60 mg/kg intraperitoneally. Since deepoxidation is a significant detoxification reaction for trichothecenes, variation in intestinal microflora among animals may account, at least in part, for the species variability in toxicity.

Biotransformation of the trichothecene mycotoxin T-2 by the hepatic S-9 fraction prepared from phenobarbital-treated rats yielded a new metabolic product designated RLM-3. The metabolite was purified from the hepatic preparation using preparative HPLC. The structural analysis of RLM-3 was carried out using gs chromatography/mass spectometry and proton and carbon-13 NMR. RLM-3 was identified as 4'-hydroxy T-2. THe toxicity of RLM-3 in comparison to T-2 toxin and 3'-hydroxy T-2 was assessed using the rat skin bicassay technique. THe metabolite 4'-hydroxy T-2 was shown to be deacylated at the C-4 position to yield 4'-hydroxy HT-2 when incubated with rat hepatic S-9 preparations.

Metabolic profiles of the excreta from rats following i.v., oral, and dermal administration of tritium-labeled T-2 toxin at 0.15 mg/kg and 0.60 mg/kg were determined. The major metabolites in urine were 3'OH HT-2, T-2 tetraol, and unknown metabolite M5, whereas the major metabolites in feces were deepoxy T-2 tetraol, 3'OH HT-2, and unknown metabolites M5, M7, and M9. The metabolite labeled M9 (major metabolite) was tentatively identified as deepoxy 3'OH HT-2. There was no significant effect on metabolic profiles due to dose, but there was a variable effect associated with the route of administration. The increase over time of appreciable levels of deepoxy metabolites as a percentage of extracted radioactivity was both consistent and statistically significant.

The enterohepatic circulation of T-2 toxin and its conjugated metabolites was examined in bile-cannulated male rats. Rats intraduodenally administered tritiated T-2 toxin eliminated 44.65% and 57.25 % of the administered dose in the bile within 4 and 8 hours post dosing, respectively. The thin-layer radiochromatographic metabolite profiles in the bile following intravenous and intraduodenal administration of T-2 toxin were similar. The major metabolites detected were 3'OH HT-2, glucuronide

conjugates, T-2 tetraol (TOL), 4-deacetylneosolaniol (4-DN) and HT-2. Tritium-labeled glucuronide conjugates obtained from the bile of rats intravenously administered [3H]T-2 toxin were extracted and purified using C-18 and silica column chromatography. The rats eliminated 6.01% and 11.86% of the dose in the bile withinin 4 and 8 hours, respectively, following intraduodenal administration of the glucuronide conjugates. No free metabolites of T-2 toxin were detected in the bile of any animals administered the purified conjugates. Oral treatment of the rats with the ß-glucuronidase inhibitor saccharic acid lactone did not produce a statistically significant decline in the amount of radioactivity recovered in the bile following administration of the glucuronide conjugates.

Metabolic and elimination profiles were investigated in rabbits following intavenous, dermal and oral administration of tritium-labeled T-2 toxin at 0.15 and 0.6 mg/kg body weight. The major metabolites detected in the urine were T-2 tetraol (TOL), deepoxy T-2 tetraol (DE TOL), 3'OH HT-2 and an unknown metabolite labeled M9. In feces, the major products eliminated were HT-2, 3'OH HT-2, DE TOL, and M9. The metabolite M9 was tentatively identified as deepoxy 3'OH HT-2. There were significant effects due to dose and route, but the effect of time was much more significant. The percentage of deepoxy metabolites compared to the total residues detected, increased steadily over time with all three routes of administration and both dosages investigated.

A gas chromatographic method for screening trichothecene mycotoxins in feeds is described. Feed is extracted with acetonitrile-water, and the toxins purified with charcoal/alumina, florisil, and silica mini-columns. Deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), T-2 toxin, and their fungal metabolites are hydrolyzed to their corresponding parent alcohols (DON, NIV, scirpentriol, or T-2 tetraol) by alkaline hydrolysis. After derivatization to their pentafluoropropionyl analogs, the toxins are quantitated by gas chromatography with electron capture detection. Confirmation and increased sensitivity can be achieved by negative chemical ionization mass spectrometry with no additional sample workup. Recoveries of DAS, DON, and T-2 toxin averaged, respectively, 80%, 65%, and 85% in corn; 84%, 65%, and 88% in soybeans; and 70%, 57%, and 96% in mixed feeds at concentrations ranging from 0.1 to 2.0 ppm. Recovery of the polar trichothecene nivalenol averaged 86% in corn. A detection limit of 0.02 ppm in corn, soybeans, and mixed feeds and 0.05 ppm in silages is estimated.

Two types of antibodies raised against T-2 toxin, namely anti-T-2-HB-BSA and anti-3-Ac-NEOS-HS-BSA, showed good cross-reactivity with deepoxy T-2 toxin. Our results indicate that the epoxide is not an important epitope for the production of antibody against T-2 toxin.

FOREWORD

Written permission has been obtained to use in this report copyrighted material from the PhD Thesis prepared by Dr. Robert H. Poppenga. Portions from his thesis are identified by those sections listing him as first author.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. [NIH] 78-23, Revised 1978).

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I. DEVELOPMENT OF PREVENTATIVE AND THERAPEUTIC REGIMENS FOR T-2 TOXICOSIS

A. Assessment of Potential Therapies for Acute T-2 Toxicosis in the Rat

by
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Abstract

The efficacy of a variety of approaches for the treatment of intravenously (iv) induced acute T-2 toxicosis was assessed utilizing young female rats. A single large dose (30 mg/kg, ip) of the water-soluble salt of methylprednisolone given 15 min after the toxin significantly prolonged survival times in iv T-2 toxin (1 mg/kg) treated animals. Other agents given ip did not prolong survival times at the dosages employed. These included diltiazem hydrochloride, dazemgrel, N-acetylcysteine, dimethyl sulfoxide, adenosine triphosphate (ATP), ATP combined with magnesium chloride (ATP-$MgCl_2$), ascorbic acid, and aprotinin (all given after the toxin) and trichodermin given either before or after the toxin. Trichodermin, a trichothecene mycotoxin like T-2, appeared to have a detrimental effect on survival time whether given 1 hr prior to or 1 hr after T-2 toxin.

Introduction

T-2 toxin [3 alpha-hydroxy-4 beta, 15-diacetoxy-8 alpha (3-methyl-butyryloxy)-12,13-epoxytrichothec-9-ene] is a secondary fungal metabolite produced by various species of Fusarium. It belongs to a larger group of mycotoxins called the trichothecenes. The trichothecenes have antifungal, antibacterial, antiviral, phytotoxic, and cytotoxic properties (Jarvis, Eppley, and Mazzola, 1983). In vitro and in vivo, T-2 toxin is a potent protein synthesis inhibitor, particularly in eucaryotic cells (Ueno et al., 1973) and is able to impair DNA synthesis (Agrelo and Schoental, 1980; Rosenstein and Lafarge-Frayssinet, 1983). In vitro, at high concentrations, T-2 toxin inhibits mitochondrial respiration (Schiller and Yagan, 1981; Pace, 1983), alters cell membrane structure and function (Chiba et al., 1972), and inactivates certain thiol-containing enzymes (Ueno and Matsumota, 1975). The relative importance of these actions for human and animal toxicity is not known. Apart from protein synthesis and, perhaps, DNA synthesis inhibition, the other effects apparently require T-2 toxin concentrations higher than those likely to be encountered in vivo.

Natural T-2 toxin production, resulting in potentially hazardous concentrations, is most likely to occur in cereal grains overwintered in the field or in improperly stored, high moisture grains. The detection of T-2 toxin in contaminated feedstuffs, although uncommon, can be associated with severe detrimental effects on human and livestock health. T-2 toxin has been implicated as 1 of the causative factors in moldy corn toxicosis of livestock (Hsu et al., 1972) and in alimentary toxic aleukia (ATA), a potentially fatal mycotoxicosis of humans and livestock (Ueno et at., 1972). The adverse health effects associated with exposure to naturally occurring T-2 toxin can be insidious, resulting from ingestion of relatively low levels of toxin over a period of time. Most often if death occurs, it follows a relatively long period of illness.

A more ominous method of exposure to T-2 toxin may be via its use as a chemical warfare agent (Haig, 1982; Schultz, 1982). T-2 toxin is suspected of being a component of "yellow rain," the popularized name given to the

chemical or chemicals allegedly used by communist forces in Southeast Asia and Afghanistan. The use of T-2 toxin as a chemical warfare agent would be expected to result in acute exposure to high levels of the toxin via inhalation or dermal contact. Oral exposure might also occur due to foodstuff and water contamination.

In experimental animals acutely exposed to T-2 toxin by the oral, intravenous, or inhalation route, a variety of organ systems are affected, especially those with a complement of rapidly dividing cells such as the gut, bone marrow, and lymphoid tissues (DeNicola et al., 1978; Brennecke and Neufeld, 1982; and Pang, 1986). Acute exposure to sufficient T-2 toxin results in the rapid onset of circulatory shock characterized by reduced cardiac output, profound arterial hypotension, lactic acidosis, and death within hours (Lorenzana et al., 1985). Topical exposure to T-2 toxin, while causing severe local tissue necrosis in rats (Hayes and Schiefer, 1979) and swine (Biehl, 1987), is not as likely to result in acute deaths. Human exposure to high levels of T-2 toxin would be expected to cause similar symptomatology and there appears to be evidence that significant mortality has followed human exposure to "yellow rain" (Haig, 1982; Shultz, 1982).

Assuming T-2 toxin was used as a chemical warfare agent, there exists a need to formulate therapeutic protocols for the treatment of acute T-2 toxicosis. Since the onset of symptoms following acute exposure to T-2 toxin can occur rapidly, treatment protocols need to be instituted quickly. Therapeutic intervention might consist initially of minimizing exposure to the toxin and preventing its systemic absorption. Once toxin is absorbed, however, general or specific therapies would need to be instituted such as symptomatic and supportive treatment, protection of specific cellular T-2 toxin binding sites, alteration of metabolism in a beneficial way, or hastening elimination of both parent compound and harmful metabolites.

There are few reports of studies designed to assess the ability of various drug agents to alleviate acute T-2 toxicosis. General supportive measures have been advocated (Committee on Protection Against Mycotoxins, 1983). Glutathione prodrugs such as the L-2-oxo and L-2-methyl derivatives of thiazolidine-4-carboxylate have been shown to increase the LD_{50} of T-2 toxin in mice (Fricke, Beauchamp, and Keeling, 1984a). In addition, the glucocorticosteroid dexamethasone improved survival in mice (Fricke, 1985) and rats (Tremel et al., 1985) given T-2 toxin. More recently, monoclonal antibodies were shown to be effective in improving survival in rats (Feuerstein, Powell, and Hunter, 1986), and ascorbic acid decreased the lethality of T-2 toxin in mice (Fricke and Jorge, 1986). The purpose of our studies was to assess a variety of agents for their efficacy in the treatment of acute T-2 toxicosis.

Materials and Methods

A series of 5 experiments was conducted, each experiment assessing a different group of agents (Table I.1). For each individual experiment, approximately 240 g female, Spraque-Dawley rats were randomly assigned to either a control or treatment group. Each individual experiment in the series included a positive control group given T-2 toxin with no therapy. In addition, where multiple drug dosings were required (Experiments 1 and 2), another positive control group was included to assess the effect of the additional handling stress on survival. This second positive control group was given sham saline injections ip as often as the most frequently handled treatment group within that particular experiment.

Upon arrival, rats were allowed to acclimate for a minimum of 7 days. Twenty-four hr prior to T-2 toxin and treatment administration, the rats were weighed and appropriate doses of T-2 toxin and the various drug agents were prepared. The rats were fasted for 12 hr prior to the start of the experiments. All animals were then dosed intravenously via the tail vein with a bolus injection of T-2 toxin at a dose of 1 mg/kg. Therapy with the various agents was begun 15 min after T-2 toxin administration with the exception of 2 treatment groups in Experiment 3, which were given trichodermin either 1 hr prior to or 1 hr after T-2 toxin administration (Table I.2).

All therapeutic agents were given ip. Dosage rates and intervals for drug agents were selected, where possible, based on a literature review of their uses in other situations. The dose of trichodermin was based on knowledge of its lower toxicity when compared to that of T-2 toxin. The sources of the agents utilized, their general mode of action, and the concentrations and vehicles used are given in Table I.1.

After dosing, all animals were returned to their cages and periodically observed. All had food and water available ad libitum after T-2 toxin administration. Survival times were recorded for those rats dying spontaneously. Animals surviving 48 hr were anesthetized with ether and exsanguinated.

A k sample test capable of handling $k > 2$ with censored observations was employed to detect overall significant differences of survival time for the groups within each experiment (Knapp and Wise, 1985). Since only improved survival was hypothesized, a 1-tailed test of significance was used. If overall significance was found, pair-wise comparisons of survival times between each treatment group and its respective control group(s) were assessed utilizing a modification of Gehan's generalized Wilcoxin test (Knapp and Wise, 1985) with the significance level for each individual comparison determined by the Bonferroni method for multiple comparisons. The proportion of animals surviving over time was calculated for those treatment groups showing significant differences compared to their control groups (Kaplan and Meier, 1958).

Results

As seen in Table I.2, the group in Experiment 1 which was given methylprednisolone sodium succinate iv at 30 mg/kg was the only treatment group with a significantly improved survival time as compared to its control group or groups. The mean survival time of the control group given only T-2 toxin was 14.10 $\pm$ .88 hr. Mean survival times for the "handling stress" and methylprednisolone treatment groups were not calculated due to censored observations. There were 3 of 8 rats in the "handling stress" control group and 6 of 8 rats in the methylprednisolone treatment group which lived for the full 48 hr observation period. There was no significant difference in survival times between the 2 control groups ($P = 0.44$). There was a significant difference between the positive control and "handling stress" control groups and the methylprednisolone treatment group ($P = .001$ and .026, respectively).

At the dosage levels employed, none of the other drugs proved to be of benefit in improving survival. The administration of trichodermin had a significant negative effect on mean survival time when given either 1 hr prior to ($P = .033$) or 1 hr after ($P = .023$) T-2 toxin administration.

Figures I.1 and I.2 graphically show the effects of methylprednisolone sodium succinate and trichodermin administration on the proportion of animals surviving over time, respectively. The median time to death can be readily determined from the graphs.

Discussion

Reasons for selecting the various test agents varied. Since a shock-like syndrome is a prominent feature of acute T-2 toxicosis, several drugs which have shown efficacy in the treatment of other shock states were evaluated. These agents included methylprednisolone sodium succinate, a glucocorticosteroid; ATP and ATP-$MgCl_2$, sources of high energy phosphate bonds; and aprotinin, a protease inhibitor.

While the use of glucocorticosteroids at high doses for the treatment of shock is still somewhat controversial, several studies have demonstrated their efficacy in the treatment of cardiogenic, endotoxic, and hemorrhagic shock (Lefer and Spath, 1977; Schumer, 1983). Reasons for their efficacy are not certain but have been postulated to be due to beneficial hemodynamic effects, maintenance of cell integrity, especially with regard to stabilization of lysosomal membranes, and certain metabolic effects such as inhibition of prostaglandin synthesis and improvement of aerobic metabolism.

Our results support earlier work in which glucocorticosteroids were shown to improve survival after administration of T-2 toxin (Fricke, 1985; Tremel et al., 1985). Methylprednisolone sodium succinate proved efficacious in our study, while dexamethasone sodium phosphate was effective in the previous reports. Tremel found that the administration of dexamethasone sodium phosphate iv to rats at a dose of 1.6 mg/kg either 30 min, 1 hr, or 3 hr after the iv injection of 0.75 mg/kg T-2 toxin (an approximate LD_{66}) significantly improved survival. Fricke demonstrated the efficacy of dexamethasone sodium phosphate in treating acute T-2 toxicosis in mice by administering the steroid at 13 mg/kg sc either 1 hr prior to, at the same time as, or at 1 or 2 hr after the administration of 5 mg/kg T-2 toxin sc (an approximate LD_{99}). All treatment groups showed decreased lethality from T-2 toxin. As a crude measure of glucocorticoid equivalency, the dose of 30 mg/kg methylprednisolone used in the present study would be approximately equal to a dose of 5.6 mg/kg dexamethasone (McEvoy, 1985).

Administration of ATP in combination with $MgCl_2$ improved survival of experimental animals in hemorrhagic and endotoxic shock and in postischemic renal or hepatic failure (Chaudry, 1985). ATP alone does not appear to be effective. The exact mechanism by which ATP-$MgCl_2$ exerts its beneficial effect is not known. Neither ATP given alone or in combination with $MgCl_2$ was found to be of benefit in improving survival in rats given a lethal dose of T-2 toxin.

Myocardial depressant factor (MDF) may play an important role in the pathophysiology of circulatory shock (Lefer, 1982). It is thought that MDF is produced by the action of proteases released from disrupted pancreatic lysosomes following pancreatic ischemia. Aprotinin appears to antagonize zymogenic and lysosomal proteases, thus decreasing MDF formation (Lefer and Barenholz, 1972). The role of MDF in the onset or exacerbation of the circulatory shock associated with acute T-2 toxicosis is unclear. It is known, however, that pancreatic blood flow is severely compromised in swine given a lethal dose of T-2 toxin (Lundeen et at., 1986) and severe pancreatic lesions have been characterized in swine given a sublethal dose of T-2 toxin

(Pang et al., 1985). Nevertheless, aprotinin, at the dose employed in our study, was not effective in improving survival.

Acute T-2 toxicosis in swine is associated with elevated levels of plasma thromboxane B_2 (TXB_2), the stable hydrolysis product of thromboxane A_2 (TXA_2) (Lorenzana et al., 1985). TXA_2 appears to have detrimental effects in other shock states including vasoconstriction and increased adhesion of platelets (Lefer, 1982). We hypothesized that the inhibition of the production of TXA_2 might improve survival. Unfortunately, dazemgel, a specific thromboxane synthetase inhibitor, did not prove to be efficacious at the dose given.

The hypothesized salutary effects of other agents used in these experiments were less well defined. Diltiazem is a slow calcium channel blocker exhibiting certain hemodynamic effects which were hypothesized to be of benefit in T-2 toxicosis. These effects include improved renal and splanchnic blood flow. It has been theorized that the heart may be a target organ of T-2 toxin (Pang et al., 1985; Yarom et al., 1983). Microscopic cardiac lesions, reported to occur in swine following T-2 toxin administration, may be compatible with excessive calcium influx into myofibers (Pang et al., 1985). The ability to block this calcium influx was theorized to lessen the effect of T-2 toxin on cardiac performance. Diltiazem, however, when given intraperitoneally did not prove beneficial in the present study. Perhaps hemodynamic measurements would better assess the efficacy of diltiazem in the treatment of acute T-2 toxicosis. Additionally, a portion of the administered dose may have been biotransformed by the liver prior to its entering the systemic circulation since approximately 60% of an orally administered dose of diltiazem is metabolized by the liver via a first pass effect (McEvoy, 1985).

T-2 toxin has been shown to deplete liver stores of glutathione (Fricke, Keeling, and Beauchamp, 1984b). N-acetylcysteine is the N-acetyl derivative of the naturally occurring amino acid L-cysteine. It is rapidly metabolized in the body to cysteine, a glutathione precursor. It has been used successfully in other toxicoses for replenishment of depleted hepatic glutathione (Rumack, 1983). Our work agrees with that of Fricke (unpublished observation) who found that the administration of N-acetylcysteine to mice was not effective in improving survival following T-2 toxin administration. It has been shown, however, that glutathione prodrugs such as the L-2-oxo and L-2-methyl derivatives of thiazolidine-4-carboxylate are beneficial (Fricke et al., 1984a). The reason for this discrepancy is not clear but may relate to the ability of the glutathione prodrugs to more readily penetrate cell membranes, thus replenishing cellular concentrations of glutathione more rapidly.

Dimethyl sulfoxide has anti-inflammatory properties and is also a scavenger of hydroxyl radicals. It has been postulated that hydroxyl radicals may play a role in the lysis of rat erythrocytes exposed to high concentrations of T-2 toxin in vitro (Segal et al., 1983). These authors found that erythrocyte lysis in the presence of T-2 toxin could be prevented by adding mannitol, a specific hydroxyl radical quencher, to the erythrocyte suspension. In our study, dimethyl sulfoxide had no beneficial effect on animal survival. Thus, the hypothesized role of T-2 toxin as an inducer of hydroxyl radical formation does not appear to play an important part in the lethality due to the toxin in vivo.

Ascorbic acid has been shown to decrease the lethality of T-2 toxin in mice (Fricke and Jorge, 1986). Animals were given T-2 toxin sc at 3.1 mg/kg (an

approximate LD_{99}) followed immediately by ascorbic acid given ip at doses ranging from 400 to 1200 mg/kg. Those mice given only T-2 toxin had 100% mortality, while mice given T-2 toxin and ascorbic acid had mortality rates between 30 and 40%. Since ascorbic acid is an antioxidant, Fricke hypothesized a beneficial effect through a reduction of lipid peroxidation secondary to free radical formation. In contrast to the above findings, we were unable to demonstrate improved survival in rats following the ip administration of ascorbic acid at a dose of 500 mg/kg 15 min after the iv administration of T-2 toxin at 1 mg/kg (an approximate LD_{99}). The lethal dose of T-2 toxin was approximately equivalent in both studies, so the reason for the discrepancy in results is not clear.

Trichodermin is a trichothecene mycotoxin with a much lower acute toxicity than T-2 toxin (Ueno, 1983). While the exact mode of action of the trichothecenes as it relates to acute toxicity is not known, it is conceivable that cell receptors may play an important role in the penetration of the cell membrane by these mycotoxins. It has been suggested that complete T-2 toxin cell membrane receptor occupation is a critical first step in the expression of T-2 cytotoxicity (Gyongyossy-Issa et al., 1985). If the difference in toxicity between T-2 toxin and trichodermin was due to different binding affinities for a common cell membrane receptor, trichodermin might have served to competitively inhibit T-2 toxin entry into the cell. In addition, both trichodermin and T-2 toxin bind and inhibit peptidyl transferase, a key enzyme in ribosomal protein synthesis (Tate and Caskey, 1973). Trichodermin has been found to compete with T-2 toxin for this site of action (Cannon, Smith, and Carter, 1976). Thus, it was also hypothesized that trichodermin might compete with T-2 toxin in much the same way that pralidoxime competes with organophosphates for binding to acetylcholinesterase. Based upon our results, however, trichodermin, given either 1 hr before or 1 hr after T-2 toxin, had no beneficial effect and, in fact, had a negative impact on mean survival time. Investigation of other possible competitive inhibitors of T-2 toxin might prove useful.

The 1 mg/kg dose of T-2 toxin used in these studies was expected to result in some survival of control animals. However, none of the control animals with the exception of 3 in the handling control group of Experiment 1 survived. This fact may have obscured some beneficial effects of those drugs which did not show efficacy with regard to survival, since survival is a relatively crude method of assessing treatment effects. T-2 toxin affects multiple organ systems and has a complex pathophysiology. It is possible that certain drugs that did not prove beneficial when assessed alone may still play an important role in therapy as part of a comprehensive treatment protocol.

Acknowledgements

The authors are grateful to Mr. Richard Manuel and Miss Renee Mariner for the excellent technical assistance. Our thanks also to Mrs. Donna Lundeen for preparing the graphs.

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able I.1 Pharmacologic actions, vehicles, drug concentrations, and sources of agents utilized.

xperiment No.	Agent	Action of Agent	Vehicle and [Drug Concentration]	Source
All	T-2 Toxin	Trichothecene mycotoxin, protein synthesis inhibitor, radiomimetic agent	50% ethanol, 50% normal saline (.9%) [1 mg/ml]	Toxicology Analytic Laboratory, University of Illinois College of Veterinary Medicine
1	Diltiazem hydrochloride	A slow calcium channel blocker	Normal saline (.9%) [10 mg/ml]	Marion Laboratories Inc.
	Methylprednisolone sodium succinate	Glucocorticosteroid	Normal saline (.9%) [20 mg/ml]	Purchased as Solu-Medrol from the Upjohn Co.
2	Dazemgrel (UK-38, 485)	Thromboxane synthetase inhibitor	50% ethanol, 50% normal saline (.9%) [10 mg/ml]	Raw material obtained courtesy of Pfizer Central Research
	N-acetylcysteine, sodium salt	Source of cysteine, the limiting amino acid in the formation of glutathione	Normal saline (.9%) [100 mg/ml]	Purchased as Mucomyst, Mead-Johnson Pharmaceutical
	Dimethyl sulfoxide	Anti-inflammatory and free radical scavenger	90% solution DMSO/10% water [300 mg/ml]	Purchased as 90% DMSO, Burlington Biomedical Corp.
3	Trichodermin	Trichothecene mycotoxin	50% ethanol 50% normal saline (.9%) [1 mg/ml]	Courtesy of Leo Pharmaceutical Products, Denmark
4	ATP ATP + $MgCl_2$ ATP-disodium salt, crystalline, from equine muscle, low calcium content	Source of high-energy phosphate	Normal saline (.9%) [200 µmol/ml] Normal saline (.9%) [ATP + $MgCl_2$ - 100 µmol ATP and 100 µmol $MgCl_2$/ml]	Purchased ATP and $MgCl_2$ from Sigma Chemical

able I.1 continued

xperiment No.	Agent	Action of Agent	Vehicle and [Drug Concentration]	Source
5	Ascorbic acid, sodium salt	Antioxidant	Sterile water [250 mg/ml]	Purchased as Scorbate, Burns-Biotec Labs
	Aprotinin--from bovine lung, affinity purified, lyophilized powder, 10-20 TIU † per mg solid	Protease inhibitor	Normal saline (.9%) [9000 KIU‡ per ml]	Purchased from Sigma Chemical

KIU = Kallikrein Inactivator Unit.
TIU = Trypsin Innibitor Unit. (1 TIU = 900 KIU)

Table I.2 Therapeutic regimens and survival data

Experiment No. and Group	Regimens	No. of Survivors to 48 hr	No. of Deaths	Mean Survival Time ± SEM (hr)	Range of Survival Times (hr)
1					
1 T-2 Control	1 mg/kg T-2 toxin iv; no therapy	0	8	14.10 ± .88	10.7-18.0
2 T-2 + Saline	.25 ml normal saline (.9%) ip each hour for 5 hr*	3	5	†	10.4-48.0
3 T-2 + Dilitiazem	18 mg/kg ip every 2 hr for 3 treatments*	0	8	13.63 ± .61	11.7-16.1
4 T-2 + Methylprednisolone	30 mg/kg given once*	6	2	†	19.6-48.0
2					
1 T-2 Control	1 mg/kg T-2 toxin iv; no therapy	0	6	16.92 ± .65	15.4-19.0
2 T-2 + Saline	.25 ml normal saline (.9%) ip every hour for 5 hr*	0	7	18.51 ± .70	17.1-21.6
3 T-2 + Dazemgrel	25 mg/kg initial dose ip followed by 10 mg/kg ip every hour for 5 hr*	0	8	16.87 ± .61	14.3-19.3
4 T-2 + N-acetylcysteine	100 mg/kg initial dose ip followed by 70 mg/kg 5 hr later*	0	6	18.18 ± .50	16.0-19.5
5 T-2 + DMSO	1 g/kg ip given once*	0	6	17.34 ± .67	14.7-19.0
3					
1 T-2 Control	1 mg/kg T-2 toxin iv; no therapy	0	8	22.75 ± 4.88	12.7-46.0
2 T-2 + Trichodermin Pretreatment	100 mg/kg trichodermin ip 1 hr prior to T-2 toxin administration*	0	8	11.47 ± 1.14	9.9-19.3
3 T-2 + Trichodermin Posttreatment	100 mg/kg trichodermin ip 1 hr after T-2 toxin administration*	0	8	10.69 ± .61	9.6-14.7
4					
1 T-2 Control	1 mg/kg T-2 toxin iv; no therapy	0	8	15.09 ± 2.71	10.5-33.9
2 T-2 + ATP	.25 ml given ip immediately and 15 minutes post-T-2 toxin*	0	8	12.41 ± .36	10.7-14.1
3 T-2 + ATP and $MgCl_2$	.125 ml given ip immediately and 15 minutes post-T-2 toxin*	0	8	14.29 ± 1.56	11.9-23.5

Table I.2 (continued)

Experiment No. and Group	Regimens	No. of Survivors to 48 hr	No. of Deaths	Mean Survival Time ± SEM (hr)	Range of Survival Times (hr)
5					
1 T-2 Control	1 mg/kg T-2 toxin iv; no therapy	0	7	12.52 ± .63	10.4-14.5
2 T-2 + ascorbic acid	500 mg/kg ip given once*	0	8	13.46 ± 1.02	10.2-18.3
3 T-2 + aprotinin	15,000 KIU/kg ‡ initial dose ip then 10,000 KIU/kg ip 2 hr later	0	8	14.35 ± 1.61	10.8-22.9

* = All treatment groups received 1 mg/kg T-2 toxin iv
† = No group means were calculated because of censored observations.
‡ = 1 KIU (Kallikrein inactivator unit) = 0.14 mcg of the crystalline active ingredient.

Figure I.1 The effect of the ip administration of methylprednisolone sodium succinate (MPSS) at 30 mg/kg immediately after the iv administration of T-2 toxin at 1 mg/kg on the proportion of rats surviving over time. The intersection of the dotted line on the X-axis estimates the median time to death for the T-2 toxin control group (13.8 hr).

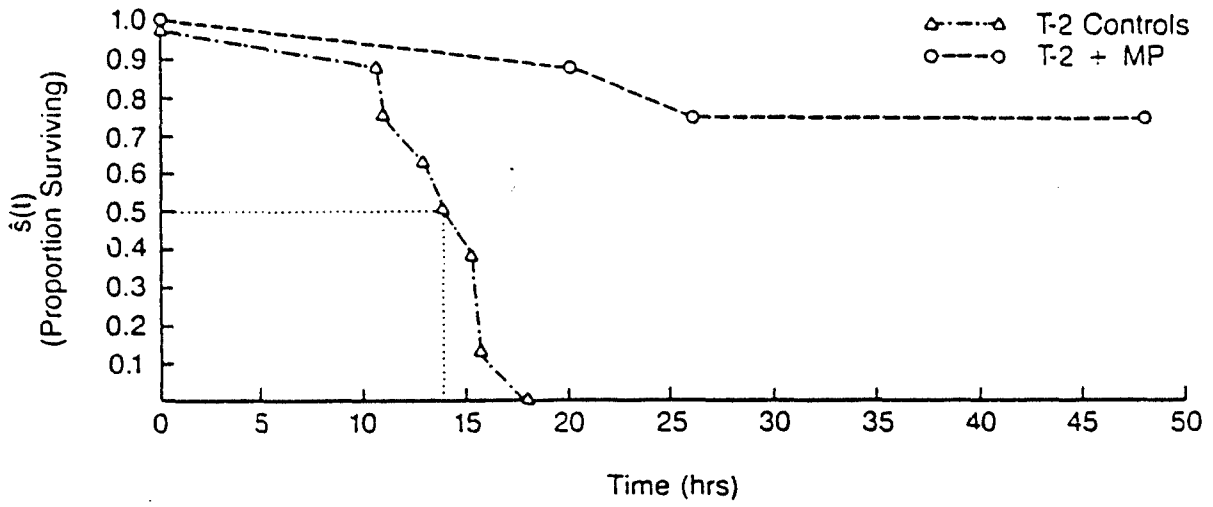
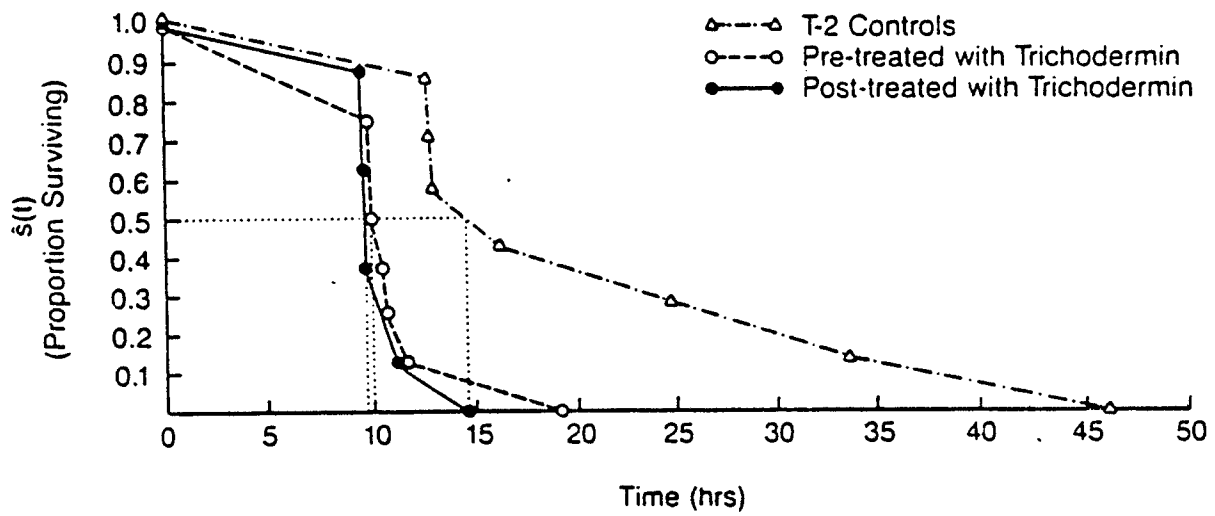


Figure I.2 The effect of the ip administration of trichodermin at 100 mg/kg given either 1 hr prior to or 1 hr after the iv administration of T-2 toxin at 1 mg/kg on the proportion of animals surviving over time. The intersections of the dotted lines with the X-axis estimate the median time to death for the post-treatment group, pre-treatment group, and T-2 toxin control group (9.8, 10.0, and 14.6 hr, respectively).



B. Therapeutic Efficacy of Orally Administered Superactivated Charcoal in Rats Exposed to a Lethal Intravenous Dose of T-2 Toxin

by
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Abstract

Studies were undertaken to assess the therapeutic efficacy of 3 superactivated charcoal oral dosing protocols for the treatment of acute parenterally induced T-2 toxicosis in rats. One gram superactivated charcoal (dry weight) per kg body weight given as a suspension in normal saline via gavage either immediately, 4, and 8 hr after the administration of T-2 toxin intravenously (iv) at 0.6 mg/kg, or immediately and 6 hr after the administration of T-2 toxin iv at 0.75 mg/kg, was not effective in improving survival rates over control groups given T-2 toxin iv (at either 0.6 or 0.75 mg/kg) followed by oral gavage with normal saline (0.9%). However, rats given 1 gram of superactivated charcoal (dry weight) per kg at 13 and 1 hr prior to and 6 hr after the administration of T-2 toxin iv at 0.8 mg/kg had significantly improved survival rates when compared to a control group given the toxin and normal saline via gavage at an identical dosing regimen as the treated rats. It appears that in rats, pretreatment with superactivated charcoal is an effective therapeutic approach for the treatment of acute T-2 toxicosis induced by iv exposure to the toxin.

Introduction

T-2 toxin (3 alpha-hydroxy-4 beta, 15-diacetoxy-8 alpha [3-methyl-butryloxy]-12,13-epoxytrichothec-9-ene) is produced by various species of Fusarium. Experimental animals acutely exposed to high intravenous (iv) doses of T-2 toxin experience circulatory shock manifested by a reduction in cardiac output, profound arterial hypotension, lactic acidosis, and death within 5 to 12 hr (Lorenzana et al., 1985; Feuerstein et al., 1985). Histologically, the most severe lesions occur in tissues with rapidly dividing cells such as the lymph nodes, spleen, thymus, bone marrow, and gastrointestinal tract (DeNicola et al., 1978; Brennecke and Neufeld, 1982; Pang et al., 1987).

Several adsorbents have been tested for their ability to bind T-2 toxin in vitro (Bratich and Buck, 1987; Fricke and Poppenga, 1987). Superactivated charcoal, which is a high surface area form of activated charcoal, exhibited the greatest degree of binding of the toxin. Due to this adsorbent capacity for the toxin, it has been extensively tested in rats and swine for treatment of orally induced acute T-2 toxicosis (Bratich and Buck, 1987; Galey et al., 1987; Fricke and Jorge, 1986; Coddington, 1986). Superactivated charcoal significantly improved survival rates when given per os (po) shortly after oral dosing with an otherwise lethal amount of the toxin. Moreover, oral administration of superactivated charcoal significantly improved survival rates of mice given T-2 toxin subcutaneously (Fricke and Jorge, 1986). Poppenga (1987) demonstrated improved survival times in swine given T-2 toxin iv when the superactivated charcoal was included in the therapeutic protocol along with other supportive therapy.

The purpose of the present study was to evaluate superactivated charcoal given po for its ability to prolong survival and prevent lethality in rats acutely exposed to T-2 toxin iv. It was felt that such an assessment, in

addition to evaluating superactivated charcoal as a possible therapeutic agent, would aid in understanding the pathophysiology of the toxicosis.

Materials and Methods

Preliminary studies were conducted to optimize the superactivated charcoal dosing protocol. In the first preliminary study, approximately 225 gram (g), female, Harlan Sprague-Dawley[1] rats were randomly assigned to 1 of 3 treatment groups (Table I.3) using a random number table (Sokal and Rohlf, 1973). Animals were allowed access to water, but not food, beginning 12 hr prior to the study. Treatment group 1 was given T-2 toxin (50% ethanol:50% saline vehicle) via a catheterized tail vein at 0.6 mg/kg followed immediately by a superactivated charcoal[2] slurry given by gavage at 1 g of superactivated charcoal (dry weight) per kg body weight. Treatment group 2 (positive control group) was given the same dose of T-2 toxin followed immediately by normal saline via gavage at a volume identical to that of the charcoal slurry. Treatment group 3 (negative control group) was given the toxin vehicle at a volume identical to that given the animals given the toxin, followed immediately by normal saline via gavage. All groups were given additional, equivalent doses of either superactivated charcoal or normal saline 4 and 8 hr after toxin administration. After dosing, the animals were offered food and water ad libitum.

Identical procedures were followed in a second preliminary study with the exception that the T-2 toxin was administered at a dose of 0.75 mg/kg and the superactivated charcoal was given immediately and 6 hr after toxin administration (Table I.4).

In Study 3, rats were randomly assigned to 1 of 3 treatment groups as before (Table I.5). Thirteen hr prior to T-2 toxin administration, all the rats were gavaged with either a superactivated charcoal slurry at 1 g (dry weight) per kg or an equivalent volume of normal saline. Food, but not water, was withheld overnight. The following morning a second, equivalent dose of either superactivated charcoal or normal saline was given by gavage. One hr later, T-2 toxin at 0.8 mg/kg body weight or an equal volume of toxin vehicle (50% ethanol:50% normal saline) was administered via a catheterized tail vein. A third dose of superactivated charcoal equivalent to the first 2 was given 6 hr after T-2 toxin administration. After dosing, the rats were offered food and water ad libitum.

In all 3 studies, the rats were periodically observed for 72 hr following treatment. Survival times for those rats dying spontaneously during the 72 hr observation period were recorded. Rats surviving for 72 hr were anesthetized with methoxyflurane[3] and exsanguinated. Since the true survival times of the latter rats could not be determined due to the scheduled euthanasia, these data were considered to be temporally censored.

A k-sample test capable of handling $k > 2$ with censored observations was used to detect overall significant differences in the survival times between the experimental groups (Knapp and Wise, 1985). Since only improved survival was hypothesized, a 1-tailed test of significance was used. If overall significance was found, a pair-wise comparison of survival times between the group given T-2 toxin and superactivated charcoal and the group given T-2 toxin and saline was assessed using a modification of Gehan's generalized Wilcoxin test (Knapp and Wise, 1985).

Results

The proportion of rats surviving in each treatment group in the first preliminary study is given in Table I.3. There was no statistically significant difference (P = .49) in the survival rates between the group given T-2 toxin followed by multiple doses of superactivated charcoal (Group 1) and the group given T-2 toxin and normal saline (Group 2). Table I.4 presents the proportion of rats surviving in each treatment group in the second preliminary study. As in the first study, there was no statistically significant difference (P = .22) between the group given T-2 toxin followed by superactivated charcoal (Group 1) and the group given T-2 toxin and no charcoal (Group 2). However, in the formal study, 8 of 15 rats pretreated with superactivated charcoal prior to being given T-2 toxin (Group 1) survived for the 72 hr observation period, whereas only 3 of 15 rats pretreated with normal saline followed by T-2 toxin (Group 2) survived for 72 hr. This was a statistically significant difference in survival rate between the 2 groups (P = .02). The proportion of rats surviving in each treatment group in the latter study is given in Table I.5 and the proportion of animals surviving over time is presented graphically in Figure I.3.

Discussion

Several early studies suggested the efficacy of adsorbents for the treatment of T-2 toxicosis in experimental animals. In rats, the inclusion of 10% bentonite to a diet which contained 3 µg T-2 toxin per g of feed was effective in overcoming the growth depression and feed refusal caused by the toxin (Carson and Smith, 1983). In a similar experiment, spent canola oil bleaching clays were found to be effective in reducing the growth depression and feed refusal in rats caused by the addition of T-2 toxin to their diet at 3 mg per kg of feed (Smith, 1984). The ability of various adsorbents to bind T-2 toxin *in vitro* has been assessed (Bratich and Buck, 1987; Fricke and Poppenga, 1987). Superactivated charcoal, with or without the addition of sorbitol and/or preservatives, appeared to have the highest capacity for binding T-2 toxin. Other adsorbents evaluated included various formulations of activated charcoal[4] and cholestyramine.

In vivo, adsorbents such as superactivated charcoal and cholestyramine are effective in preventing lethality following the administration of acutely toxic doses of T-2 toxin po, especially when given shortly after exposure to the toxin. Superactivated charcoal given by gavage at 1 g charcoal (dry weight) per kg body weight enhanced survival times and survival rates in rats given T-2 toxin po at 8 mg/kg (an approximate 1.5 X LD_{50}) as late as 3 hr after toxin administration (Galey et al., 1987). There was some evidence that the survival rate could be improved over control values when the superactivated charcoal was given as late as 5 hr after the toxin although the difference in survival rates was not statistically significant. Fricke and Jorge (1986) challenged mice with T-2 toxin po at 5 mg/kg followed by either superactivated charcoal at 7 g/kg or equivalent volumes of water, immediately or 1 hr after toxin administration. The percentage of surviving mice in the charcoal treated groups was significantly higher than the untreated controls, with 100% and 70% survival rates for those groups given charcoal immediately and 1 hr after the toxin, respectively. Coddington (1986) showed the effectiveness of giving superactivated charcoal po at 1 g charcoal (dry weight) per kg immediately after the oral administration of T-2 toxin po at 10 mg/kg (an approximate 2 X LD_{50}) for ameliorating the signs of acute T-2 toxicosis and preventing death in swine.

Interestingly, the administration of either superactivated charcoal (Fricke and Jorge, 1986) or cholestyramine (Fricke and Poppenga, 1987) decreased the lethality of T-2 toxin administered parenterally to mice. Fasted mice were given T-2 toxin subcutaneously at 2.8 mg/kg followed by either superactivated charcoal or cholestyramine po at 7 g/kg. The respective mortality rates for control, charcoal treated, and cholestyramine treated groups were 50%, 10%, and 20%, respectively. This suggested the possibility that a portion of the administered toxin was reaching the gastrointestinal tract as parent compound and/or toxic metabolite(s) and that the charcoal was adsorbing the parent or metabolized toxin, thus limiting further systemic or local effects.

T-2 toxin is rapidly and extensively metabolized by the liver and a large portion of the administered toxin is eliminated in the bile of guinea pigs (Pace et al., 1985) and swine (Corley et al., 1985). Corley et al. (1986) demonstrated that an average of 77% of the metabolites in the bile of swine given tritium-labelled T-2 toxin iv at 0.15 mg/kg was present as glucuronide conjugates. Thus, approximately 23% of the total metabolite residues in the gastrointestinal tract should not have been conjugated and therefore extractable. While such an extraction efficiency was found for metabolites in the stomach and small intestines, up to 77% of total metabolites in the large intestine were extractable. This provides presumptive evidence in swine for intestinal microflora-induced hydrolysis of glucuronide conjugates of T-2 toxin and its metabolites excreted in the bile. There is substantial evidence that many xenobiotics and their metabolites are eliminated in the bile as glucuronide conjugates, deconjugated in the intestinal tract by intestinal microflora, and undergo enterohepatic recirculation (Renwick, 1986). Matsumoto et al. (1978) observed a biphasic increase in blood radioactivity in mice following the administration of tritium-labelled T-2 toxin po at 1 mg/kg. This also suggests that T-2 toxin and/or its metabolites undergo enterohepatic recirculation, although more definitive studies are lacking.

Although the superactivated charcoal-associated reduction in the toxicity of parenterally administered T-2 toxin is probably related to adsorption of the toxin or its metabolites, it is also possible that the charcoal is binding endotoxin, thereby preventing its systemic absorption through a compromised intestinal barrier. The role that endotoxin plays in the pathophysiology of acute T-2 toxicosis is not clear. However, the gastrointestinal tract is a target organ of T-2 toxin as is evidenced by the severe damage to the gastric mucosa and the intestinal crypt epithelial cells, especially along the distal portions of the small intestine and the cecum (DeNicola et al., 1978; Brennecke and Neufeld, 1982; Pang et al., 1987). Brennecke and Neufeld (1982) reported histologic evidence of an overgrowth of cecal bacteria associated with severe cecal epithelial necrosis in rats given T-2 toxin im, sc, or ip at 0.47, 0.56, and 2.18 mg/kg, respectively (all are approximate LD_{50} doses). They speculated that endotoxin elaboration by the bacteria could contribute to lethality following toxin exposure. Activated charcoal can effectively bind endotoxin (Cooney, 1980). It may also be possible that a combination of the enterohepatic circulation of T-2 toxin and/or its metabolites and an increase in either the production or systemic absorption of endotoxin may be contributing to the toxicosis.

Our preliminary studies focused on optimizing the administration of T-2 toxin and the superactivated charcoal. We attempted to find a dose of T-2 toxin which would cause a significant mortality rate (approximately 75%) in untreated rats but which would not cause 100% mortality. Our initial doses

of 0.6 and 0.75 mg/kg resulted in mortality rates below 50%. Thus the dose of 0.8 mg/kg was selected for the third study.

A commonly recommended dose of 1 g activated charcoal (on a dry weight basis) per kg body weight was used in all 3 studies (Buck and Bratich, 1986). The timing of the doses was empiric. In the preliminary studies, when the superactivated charcoal was given following toxin administration, no improvement in survival rates occurred over that obtained without therapy. Routine postmortem examinations of all animals showed that the superactivated charcoal was being retained primarily in the stomach and proximal small intestine even up to 24 hr following charcoal administration. This suggests that the toxin can cause intestinal ileus. Evidence to support this comes from the observation that fusarenon-x, another trichothecene mycotoxin with a similar structure and biochemical activity to that of T-2 toxin, given to rats iv at 1 mg/kg inhibited intestinal peristalsis (Matsuoka et al., 1979). Galey et al. (1987) reported that superactivated charcoal given po following the oral administration of T-2 toxin was not found beyond the jejunum in rats that died within 24 hr. Therefore, toxin-induced inhibition of intestinal peristalsis may prevent the superactivated charcoal from reaching aboral portions of the intestine where more of the unconjugated toxin and metabolites would likely be present. This may be particularly important in the large intestine where most beta-glucuronidase necessary for deconjugation is found (Renwick, 1986). Deconjugation would likely result, not only in increased availability of toxin and metabolites for reabsorption, but also (due to a reduction in polarity from deconjugation) may cause the toxin to become more readily bound by the charcoal if present.

Because of the delay in charcoal transit through the gastrointestinal tract, the pretreatment dosing protocol was evaluated. Activated charcoal was noted in the feces of most animals pretreated with the charcoal by the time of toxin administration 13 hr later. Postmortem examinations revealed a good distribution of activated charcoal throughout the tract in these animals.

In summary, gastrointestinal adsorbents, especially superactivated charcoal, are an effective therapeutic approach for the prevention or treatment of acute T-2 toxicosis in experimental animals. Provided an optimal adsorbent to toxin ratio is maintained, superactivated charcoal, if given immediately after toxin exposure, appears to be nearly 100% effective in preventing death following oral exposure to otherwise lethal doses of T-2 toxin. Superactivated charcoal also improves survival following parenteral exposure to the toxin, although in rats, pretreatment appears to be necessary in order to allow enough of the adsorbent to reach more aboral segments of the gastrointestinal tract where it may effectively bind the toxin or its metabolites.

Acknowledgements

The authors are grateful to Barbara Kindler, Mary Busse, and Tina Keferlis for their excellent technical assistance. Our thanks also to Donna Lundeen for preparing the graph and Sally Campbell for typing the tables.

These studies were supported in part by the U.S. Army Medical Research and Development Command, Contract No. DAMD 17-82-C-2179 and 17-85-C-5224. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed and an official Department of the Army position, policy, or decision unless so designated by other documentation.

Footnotes

[1]Harlan Sprague Dawley, Inc., Indianapolis, IN.

[2]SuperChar●, Gulf-Biosystems, Inc., Dallas, TX.

[3]Metofane●, Pitman-Moore, Inc., Washington Crossing, NJ.

[4]Calgon Activated Carbon●, Calgon Corp., Pittsburgh, PA.
Toxiban●, Vet-A-Mix, Inc., Shenandoah, IA.
Norit A●, American Norit Co., Inc., Jacksonville, FL.

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Table I.3 Superactivated charcoal preliminary study 1. Treatment groupings, dosing protocols, and survival data.

Treatment Group	Dosing Protocol	No. of Survivors to 72 hr / Total No. of Animals in Group
1. T-2 toxin + superactivated charcoal	0.6 mg/kg T-2 toxin* iv* followed immediately, 4, and 8 hr later by a superactivated charcoal slurry*** via gavage at 1 g charcoal/kg bw.	7/10
2. T-2 toxin + normal saline	0.6 mg/kg T-2 toxin* iv** followed immediately, 4, and 8 hr later by normal saline via gavage at a volume equivalent to the activated charcoal slurry***.	7/10
3. T-2 toxin vehicle + normal saline	50% ethanol:50% normal saline mixture iv** at an equivalent volume to the T-2 toxin dose followed immediately, 4, and 8 hr later by normal saline via gavage at a volume equivalent to the activated charcoal slurry.	10/10

*T-2 toxin was dissolved in 50% ethanol:50% normal saline to a final concentration of 0.6 mg toxin per ml of vehicle.

**All iv injections were via a catheterized tail vein.

***100 mg superactivated charcoal per ml of normal saline.

Table I.4 Superactivated charcoal preliminary study 2. Treatment groupings, dosing protocols, and survival data.

Treatment Group	Dosing Protocol	No. of Survivors to 72 hr / Total No. of Animals in Group
1. T-2 toxin + superactivated charcoal	0.75 mg/kg T-2 toxin* iv** followed immediately and 6 hr later by a superactivated charcoal slurry*** via gavage at 1 g charcoal/kg bw	4/7
2. T-2 toxin + normal saline	0.75 mg/kg T-2 toxin* iv** followed immediately and 6 hr later by normal saline via gavage at a volume equivalent to the activated charcoal slurry***	5/7
3. T-2 toxin vehicle + normal saline	50% ethanol:50% normal saline mixture iv** at an equivalent volume to the T-2 toxin dose followed immediately and 6 hr later by normal saline via gavage at a volume equivalent to the activated charcoal slurry.	7/7

*T-2 toxin was dissolved in 50% ethanol:50% normal saline to a final concentration of 0.75 mg toxin per ml of vehicle.

**All iv injections were via a catheterized tail vein.

***100 mg superactivated charcoal per ml of normal saline.

Table I.5 Superactivated charcoal study 3. Treatment groupings, dosing protocols, and survival data.

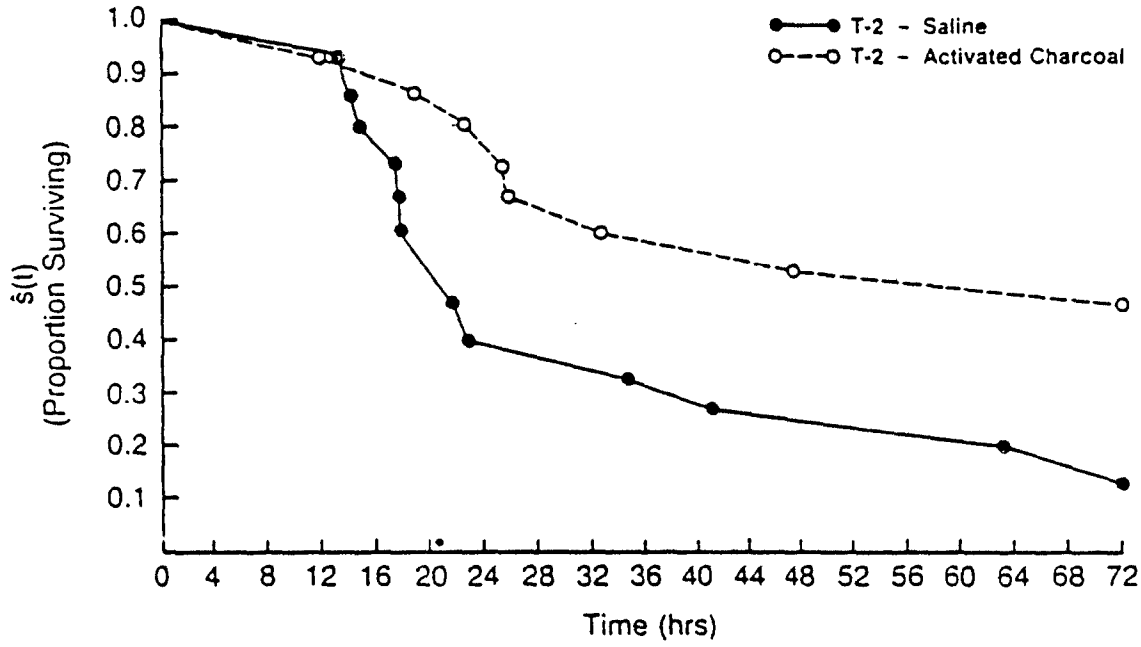
Treatment Group	Dosing Protocol	No. of Survivors to 72 hr / Total No. of Animals in Group
1. T-2 toxin + superactivated charcoal	Superactivated charcoal slurry*** via gavage at 1 g/kg bw 13 hr and 1 hr prior to and 6 hr after T-2 toxin* iv** at 0.8 mg/kg.	8/15
2. T-2 toxin + normal saline	Normal saline via gavage at a volume equivalent to the superactivated charcoal slurry 13 hr and 1 hr prior to and 6 hr after T-2 toxin* iv** at 0.8 mg/kg	3/15
3. T-2 toxin vehicle + normal saline	Normal saline via gavage at a volume equivalent to the superactivated charcoal slurry*** 13 hr and 1 hr prior to and 6 hr after 50% ethanol:50% normal saline mixture iv** at equivalent volumes to the T-2 toxin dose	12/12

*T-2 toxin was dissolved in 50% ethanol:50% normal saline to a final concentration of 0.8 mg toxin per ml of vehicle.

**All iv injections were via a catheterized tail vein.

***100 mg superactivated charcoal per ml of normal saline.

Figure I.3 Effect of treatment with superactivated charcoal on the proportion of rats surviving over time. The symbol along the X-axis indicates the approximate median survival time for the group given T-2 toxin + saline.



C. The Evaluation of Therapeutic Intervention on Histologic Tissue Changes Following Intravenous Administration of T-2 Toxin in Rats

by
Robert H. Poppenga, Val R. Beasley, and William B. Buck

1. Intravenous therapy with methylprednisolone sodium succinate

Abstract

A semiquantitative scoring system was used to assess changes in the severity of histologic tissue lesions in rats given methylprednisolone sodium succinate (MPSS) ip at 30 mg/kg 15 min after the administration of T-2 toxin iv at 1.0 mg/kg. Control animals given T-2 toxin but no therapy developed histologic lesions characteristic of acute T-2 toxicosis in lymphoid tissues, the gastrointestinal tract, pancreas, and adrenals. There was a significant decrease in the severity of tissue lesions in the glandular portion of the stomach and the spleen in the group treated with MPSS compared to the positve control group. In a group given MPSS alone, lymphocyte necrosis was noted in the cortex of the thymus. Therapy had no effect on the severity of tissue lesions in the duodenum, jejunum, ileum, cecum, pancreas, or adrenals.

Introduction

The trichothecene mycotoxin [3 alpha-hydroxy-4 beta, 15-diacetoxy-8 alpha (3-methyl-butryloxy)-12, 13-epoxytrichothec- 9-ene], better known by its trivial name T-2 toxin, is a secondary fungal metabolite produced by several Fusarium species. Naturally occurring T-2 mycotoxicosis involving both man and animals has been reported in several parts of the world (Hsu et al., 1972; Puls and Greenway, 1976; Ueno et al., 1972; Yagen and Joffe, 1976).

In vitro and in vivo, T-2 toxin is a potent protein synthesis inhibitor in eucaryotic cells (Ueno et al., 1973) and is able to impair DNA and RNA synthesis (Agrelo and Schoental, 1980; Rosenstein and Lafarge-Frayssinet, 1983). At various higher concentrations, T-2 toxin alters cell membrane structure and function (Chiba et al., 1972; Trusal, 1985). In vitro, T-2 toxin inactivates certain thiol-containing enzymes (Ueno and Matsumota, 1975) and has vasoactive action (Wilson and Gentry, 1985). T-2 toxin also depresses hepatic glutathione concentrations in vivo (Fricke et al., 1984). The relative importance of these actions for human and animal toxicity is not known but it is necessary to recognize the fact that protein synthesis inhibition is the most potent effect of the toxin.

Acute T-2 toxicosis is characterized by a circulatory shock syndrome in swine, rats, and guinea pigs (Beasley et al., 1986; Lorenzana et al., 1985; Feuerstein et al., 1985). Clinically, the shock is evidenced by declines in cardiac output and mean aortic blood pressure and the occurrence of lactic acidosis. Additionally, in swine, absolute blood flow to vital organs such as the heart and brain and less vital organs such as the spleen and pancreas is significantly impaired (Lundeen et al., 1986).

Histologic changes after an acutely toxic dose of T-2 toxin, administered by a variety of routes, have been characterized in a number of experimental animal species (Sato et al., 1975; DeNicola, et al., 1978;

Weaver et al., 1978; Hoerr et al., 1981, Brennecke and Neufeld, 1982; Thurman et al., 1986; Pang et al., 1987). Consistent histologic changes include severe lympholysis in lymphoid tissues such as lymph nodes, thymus, and spleen and severe congestion and necrosis of the gastrointestinal mucosa particularly the glandular portion of the stomach and proximal portions of the small intestine. Multifocal degeneration of single or grouped pancreatic acinar cells in iv dosed swine and adrenal parenchymal cell necrosis in female mice exposed by inhalation also occur (Pang et al., 1987; Thurman et al., 1986).

The most effective therapies for acute, experimental T-2 toxicosis in animals include iv administration of high doses of the water-soluble glucocorticosteroids, methylprednisolone sodium succinate (MPSS) and dexamethasone sodium phosphate (Fricke, 1985; Tremel et al., 1985; Poppenga et al., 1987), or oral administration of intestinal adsorbents such as superactivated charcoal or cholestyramine (Galey et al., 1987; Fricke and Poppenga, 1987; Fricke and Jorge, 1986).

The objectives of this report are to: 1) describe the efficacy of MPSS for preventing histologic lesions in certain target organs in rats given an otherwise lethal iv dose of T-2 toxin and 2) to discuss the significance of the findings with regard to survival following therapy.

Materials and Methods

Animals

Female, Harlan Sprague-Dawley rats weighing approximately 240 g were allowed to acclimate to their environment for a minimum of 7 days prior to inclusion in the study. They were fed a standard ration which contained no detectable concentrations of the trichothecene mycotoxins, T-2 toxin, diacetoxyscirpenol, and vomitoxin or the non-trichothecene mycotoxins, zearalenone and aflatoxin.

Toxin and Drug Agent

Purified T-2 toxin was obtained from the Toxicologic Analytical Laboratory, University of Illinois College of Veterinary Medicine. The toxin purity was $\geq$ 95% assessed by GC using a FID detector. The toxin was dissolved in 50% ethanol:50% saline to a concentration of 1 mg/ml.

Methylprednisolone sodium succinate was purchased as Solu-Medrol from the Upjohn Co. The sterile powder was dissolved in normal saline to a concentration of 20 mg/ml.

Experimental Protocol

Twenty-four hr prior to the start of the experiment, the rats were weighed and randomly assigned to 1 of 4 experimental groups as follows: Group 1 (n = 7) was given T-2 toxin iv at 1 mg/kg followed 15 min later by normal saline given ip, Group 2 (n = 7) was given T-2 iv at 1 mg/kg followed 15 min later by MPSS given ip at 30 mg/kg (same volume per kg as saline), Group 3 (n = 3) was given normal saline iv followed 15 min later by MPSS at 30 mg/kg, and Group 4 (n = 3) was given normal saline iv followed 15 min later by normal saline ip. Rats were fasted for 12 hr prior to dosing. All iv injections were given as a bolus via a tail vein.

After dosing, all rats were returned to their cages and periodically observed. After administration of T-2 toxin and therapy, the rats had food and water available ad libitum. Rats in Groups 2, 3, and 4 were dosed 2 months after those in Group 1 and were killed 14 hr after treatment administration to serve as time matched controls to those in Group 1 (mean survival time 14.10 ± 0.88).

Tissue specimens were taken from all animals immediately following death and fixed in 10% neutral buffered formalin. After fixation, the tissues were embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin (HE), and examined by light microscopy. Target tissues examined included the thymus, spleen, glandular portion of the stomach, duodenum, jejunum, ileum, cecum, pancreas, and adrenal.

To determine whether there was a treatment effect on the severity of T-2 toxin-induced lesions, an evaluation of the microscopic changes present was performed using a semiquantitative scoring system modified from Coppock et al. (1985). The total lesion score for the spleen was the sum of the products of the estimated number of follicles involved (B-cell areas) x the score for percentage lympholysis and the estimated number of periarteriolar cuffs involved (T-cell areas) x the score for percent lympholysis. The estimated number of splenic follicles or periarteriolar cuffs involved was scored as: 0 = normal background, 1 = occasional, 2 = most, and 3 = all. The severity of lymphoid necrosis as a percentage of lymphoid cells which were necrotic was scored from 0 to 4 as follows: 0 = normal background, 1 = ≤ 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%. The possible scores ranged from 0 to 24. The total lesion score for the thymus was the sum of the scores for severity of lymphoid necrosis of the cortex and the medulla with the severity of lymphoid necrosis scored as for the spleen. The possible scores ranged from 0 to 8. Intestinal-associated lymph tissue lesions were scored as the product of the number of follicular areas involved and the severity of lymphoid necrosis within germinal centers. Possible scores ranged from 0 to 12. The scoring criteria were the same as those for the spleen. There were too few observations for statistical analysis of the data for intestinal-associated lymph tissue.

The total lesion score for the stomach was the sum of the degree of congestion/hemorrhage and degree of necrosis scored as: 0 = normal background, 1 = mild, 2 = moderate, and 3 = severe. The possible scores ranged from 0 to 6. The total lesion scores for the different segments of the intestinal tract were the product of the percentage of intestinal crypts affected and the percentage of epithelial cell necrosis within individual crypts with each scored from 0 to 4 as follows: 0 = normal background, 1 = 10 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%. The possible scores ranged from 0 to 16.

The total lesion score for the adrenal was the sum of the degree of congestion/hemorrhage and degree of necrosis and inflammatory cell infiltration scored as: 0 = normal background, 1 = slight, 2 = mild, 3 = moderate, and 4 = severe. The possible scores ranged from 0 to 8. The total lesion score for the pancreas was the product of the number of acini involved scored as 1 = normal background, 2 = occassional, and 3 = many; and the number of necrotic cells within each acinus scored as: 1 = normal background, 2 = up to 1/3, 3 = 1/3 to 2/3, and 4 > 2/3. The possible scores ranged from 0 to 12.

Statistical Analysis

Differences between the positive control group and the time-matched MPSS treatment group for each tissue were assessed by the Kruskal-Wallis 1-way analysis of variance by ranks (Kruskal and Wallis, 1952) using a computer statistical package. A probability level equal to or less than 5% ($p \leq 0.05$) was considered significant.

Results

In the positive control group, histologic lesions noted in the tissues examined were characteristic of acute T-2 toxicosis. Histologic lesions in the spleen were characterized by severe generalized congestion and hemorrhage and cell depletion in both follicular (B-cell) and periarteriolar (T-cell) regions. The most severe lympholysis occurred in splenic follicular germinal centers. Marked multifocal lympholysis occurred in both the cortex and medulla of the thymus.

The gastric lesions were restricted to the glandular region and were characterized as a necrotizing, hemorrhagic gastritis. In general, necrosis predominated in the lower half of the mucosa with congestion and/or hemorrhage more evident in the upper half. Enteric lesions consisting of pyknosis, karyorrhexis, and necrosis of crypt enterocytes occurred. The basal portions of the crypts were often filled with cellular debris and the lamina propria contained numerous inflammatory cells. In general, the lesions were more severe in the duodenum and jejunum and less severe in the ileum and cecum.

The pancreas was relatively unaffected, although individual acinar cell degeneration and necrosis occurred. The islet cells were unaffected. The zona fasiculata of the adrenal gland had areas of congestion and hemorrhage; inflammatory cell infiltration; and small, multifocal areas of parenchymal cell necrosis. In addition, inflammatory cell infiltration was noted in the zona glomerulosa.

The raw scores for lesion severity for each tissue examined and their corresponding ranks are given in Table I.6. In rats given T-2 toxin + MPSS, only the spleen and the glandular portion of the stomach were less severely affected compared to the positive controls given T-2 toxin and no therapy ($p = .005$ and .009, respectively). No significant decrease in lesion severity was noted for the thymus, the various gastrointestinal segments, the pancreas or the adrenal gland.

In the stomach, there was a marked decrease in congestion and hemorrhage in the rats given T-2 toxin + MPSS as compared to the positive control group given T-2 toxin + saline. In addition, there was a marked decrease in the number of necrotic crypt cells. Figs. I.4 through I.6 illustrate representative differences among the 3 treatment groups with regard to the histologic appearance of the stomach.

Histologically, the differences in lesion severity for the spleen were a matter of degree. Compared to negative controls, the spleens of saline + MPSS-treated rats were significantly congested and/or hemorrhagic. There was also marked lympholysis in the germinal centers of the splenic follicles and a decrease in cellularity in other areas. While not statistically evaluated, there did not appear to be any differences in the degree of lympholysis in the germinal centers of intestinal-associated

lymphoid tissues. Figs. I.7 through I.9 illustrate representative differences among the 3 treatment groups with regard to the histologic appearance of the spleen.

While there was no statistically significant difference in lesion severity for the thymus, the scores for the group given T-2 toxin + MPSS tended to be higher than those for the positive control group (Table I.6). Of additional interest was the degree of multifocal lympholysis noted in the thymic cortices of the group given saline + MPSS. Figs. I.10 through I.12 illustrate representative differences among the 3 treatment groups with regard to the histologic appearance of the thymus.

Discussion

The water-soluble salt of MPSS is effective in reducing mortality associated with circulatory shock states, particularly endotoxic shock (Shatney, 1982; Lefer and Spath; 1984). MPSS has also been shown to improve survival in rats given an otherwise lethal dose of T-2 toxin (Poppenga et al., 1987). The mechanism of beneficial action of MPSS and other water-soluble glucocorticosteroids for the treatment of circulatory shock is hypothesized to be due primarily to stabilization of cell membranes with a secondary reduction in the release or production of potentially harmful substances such as lysosomal enzymes and myocardial depressant factor, respectively (Shatney, 1982). In addition, there is some evidence that glucocorticosteroids may improve microcirculation in shock states (Altura and Altura, 1974).

Gastric lesions occur in hemorrhagic and endotoxic ciculatory shock (Harjora and Sivula, 1966; Itoh and Guth, 1985). It has been hypothesized that circulatory shock-induced gastric lesions are due to mucosal ischemia and hypoxia which may result in the production of oxygen-derived free radicals (Cheung et al., 1976; Itoh and Guth, 1985). Additionally, compromised blood flow may decrease the clearance of back-diffusing hydrogen ions which may also contribute to mucosal damage (Kivilaakso et al., 1978). In a hemorrhagic shock model using dogs, the administration of MPSS iv at 30 mg/kg ameliorated the mucosal ischemia and histologic lesions which occurred in control animals (Bowen, 1979). Whether this was a primary or secondary effect of the glucocorticoid is not known, although protection against mucosal alterations has been associated with an improvement in gastric blood flow (Ritchie et al., 1978).

Gastric lesions have also been noted to occur in experimental animals acutely exposed to high doses of T-2 toxin (DeNicola et al., 1978; Pang et al., 1987). The pathogenesis of these lesions is not certain but may relate to a marked decline in gastric blood flow which has been demonstrated in swine given T-2 toxin iv (Beasley et al., 1987). The results of the present study demonstrate that MPSS is effective in reducing the severity of T-2 toxin-induced gastric lesions.

We hypothesize that gastric lesions associated with acute T-2 toxicosis are due to mucosal ischemia and hypoxia. The ischemia prevents the removal of back-diffusing hydrogen ions which causes intracellular acidosis. Hypoxia results in the production of free radicals causing cell membrane disruption and release of locally acting substances such as lysosomal enzymes. These further aggravate mucosal damage. MPSS is able to stabilize cell membranes and preserve the mucosal microcirculation

thus prolonging cell viability and interupting the cycle of ischemia, hypoxia, and cell destruction.

Although there was a statistically significant decrease in the severity of splenic lesions, the T-2 toxin + MPSS treatment group still had marked splenic involvement. The lesions in this treatment group were characterized by generalized congestion, hemorrhage, cell depletion, and lympholysis in follicular and periarteriolar areas. Thus, MPSS did not eliminate splenic alterations. The reason for the amelioration of histologic changes in the spleens of those rats given T-2 toxin + MPSS is not clear.

Thymic lesions in the MPSS treatment group were at least partially a result of drug therapy. All 3 rats in the group given saline + MPSS had multifocal areas of cortical lympholysis which were not found in the 3 rats given saline alone. It is well known that cortisone and other glucocorticoids cause lysis of the small, non-blastogenic lymphocytes of the thymus within hr of administration (Metcalf, 1966). The release of endogenous steroids due to the stress of the toxicosis may also contribute to lymphocyte damage. Although the mechanism of glucocorticoid-induced lympholysis is not known for certain, it may relate to an influx of calcium into the cell (Kaiser and Edelman, 1977). There did appear to be a qualitative indication of an additive effect of T-2 toxin and MPSS on the degree of cortical lympholysis even though there was not a statistically significant difference in lesion scores between the group given T-2 toxin + saline and the group given T-2 toxin + MPSS.

The reason for the lack of effect of MPSS on the other tissues examined, particularly the intestinal tract, is not clear. It may be that the toxin has a direct effect on cell components of enteric epithelial cells which cannot be ameliorated by glucocorticosteroid administration. The possibility of enterohepatic recirculation of microbially deconjugated toxic metabolites may prolong the exposure of these cells to the toxin and its metabolites. The enhanced survival in rats given MPSS as reported elsewhere (Poppenga et al., 1987) may therefore result from a prevention of secondary damage to adjacent, non-target cells and prevent a positive feedback loop eventually leading to multiorgan failure and death.

In summary, it was anticipated that the beneficial action of MPSS for treating acute T-2 toxicosis would be reflected histologically by a decrease in cell destruction, particularly in those tissues most susceptible to the effect of the toxin. However, in the present study, there was a significant reduction in lesion severity only for the glandular portion of the stomach and the spleen.

Acknowledgements

The authors are grateful to Dick Manuel for his excellent technical help. Our thanks also to Laura Beachy for typing the mauscript and table.

These studies were supported in part by the U.S. Army Medical Research and Development Command, Contract No. DAMD 17-82-C-2179 and 17-85-C-5224. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

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able I.6 Raw scores and corresponding ranks of lesions from tissues examined histologically.

Treatment group	Tissue																	
	Thymus		Spleen		Stomach		Duodenum		Jejunum		Ileum		Cecum		Pancreas		Adrenal	
-2 Toxin + Saline Animal ID	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R
4T2C	2	4.0	18	8.5	4	13.5	16	11.0	N/A	--	N/A	--	N/A	--	N/A	--	7	9.0
6T2C	1	2.0	34	13	4	13.5	12	6.5	8	4.0	8	6.5	4	4.5	2	4.5	N/A	--
24T2C	1	2.0	21	11	3	11.0	N/A	--	N/A	--	12	9.0	N/A	--	N/A	--	7	9.0
25T2C	6	10.0	24	13	2	8.0	12	6.5	12	7.5	4	4.0	4	4.5	1	1.5	4	1.5
36T2C	4	8.5	18	8.5	1	4.5	12	6.5	12	7.5	3	1.5	4	4.5	3	8.0	7	9.0
38T2C	1	2.0	18	8.5	3	11.0	16	11.0	8	4.0	12	9.0	N/A	--	3	8.0	7	9.0
48T2C	N/A	--	15	4.5	3	11.0	N/A	--	12	7.5	4	4.0	4	4.5	N/A	--	7	9.0
Rank Sum		28.5		67		72.5		41.5		30.5		34.0		18.0		22.0		46.5
-2 Toxin + MPSS																		
3	3	6	24	13	2	8.0	8	2.5	4	1.5	3	1.5	4	4.5	4		4	1.5
9	4	8.5	9	1	1	4.5	4	1.0	4	1.5	N/A	--	2	1.0	2	4.5	N/A	--
11	3	6	15	4.5	0	1.5	16	11.0	16	11.0	N/A	--	8	10.0	2	4.5	6	5.5
15	3	6	15	4.5	1	4.5	12	6.5	16	11.0	4	4.0	8	10.0	4	10.0	8	12.0
17	8	11.5	15	4.5	2	8.0	12	6.5	12	7.5	8	6.5	4	4.5	3	8.0	5	3.5
19	8	11.5	18	8.5	0	1.5	12	6.5	16	11.0	12	9.0	6	8.0	1	1.5	5	3.5
22	N/A	--	14	2	1	4.5	8	2.5	8	4.0	16	11.0	8	10.0	2	4.5	6	5.5
Rank Sum		49.5		38*		32.5*		36.5		47.5		32.0		48.0		44.0		31.5
	p = .087		p = .005		p = .009		p = .115		p = .737		p = .709		p = .211		p = .695		p = .210	

RS = Raw Score
R = Rank
/A = Tissue not available for evaluation
* = Significantly different from control group ($p \leq .05$)
-- = No rank assigned due to missing tissues

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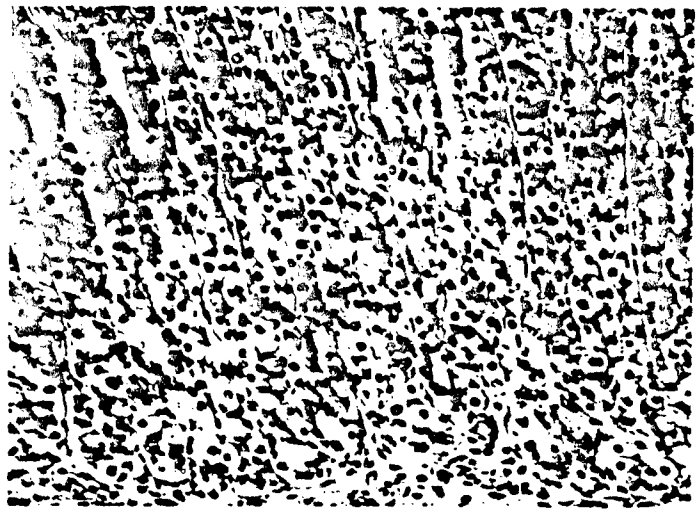
Figure I.4 Stomach from a rat given saline iv at a volume equivalent to the T-2 toxin dose + MPSS ip at 30 mg/kg and killed 14 hr later. There are no histologic lesions present. (H&E). 62.5 X

Figure I.5 Stomach from a rat given T-2 toxin iv at 1 mg/kg + MPSS ip at 30 mg/kg and killed 14 hr later. There are isolated, necrotic cells within the gastric glands (arrows). There are a number of mononuclear cells within the mucosal capillaries (arrowhead). (H&E). 62.5 X

Figure I.6 Stomach from a rat given T-2 toxin iv at 1 mg/kg + saline ip at a volume equivalent to the MPSS dose. There is widespread necrosis of individual cells with the gastric glands (arrows). (H&E). 62.5 X

4



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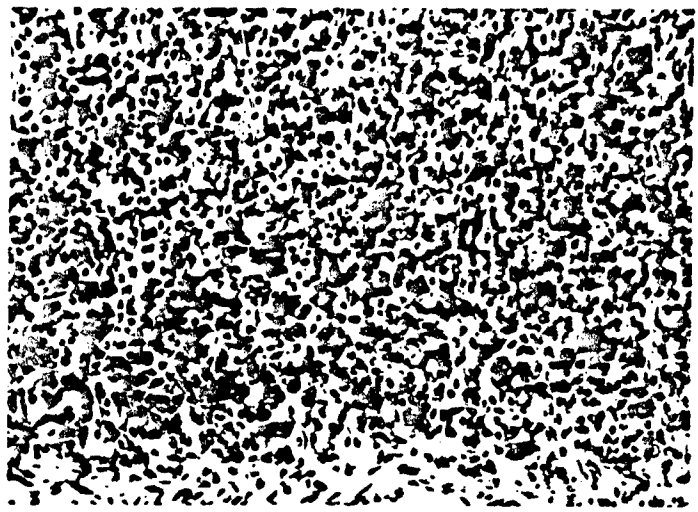

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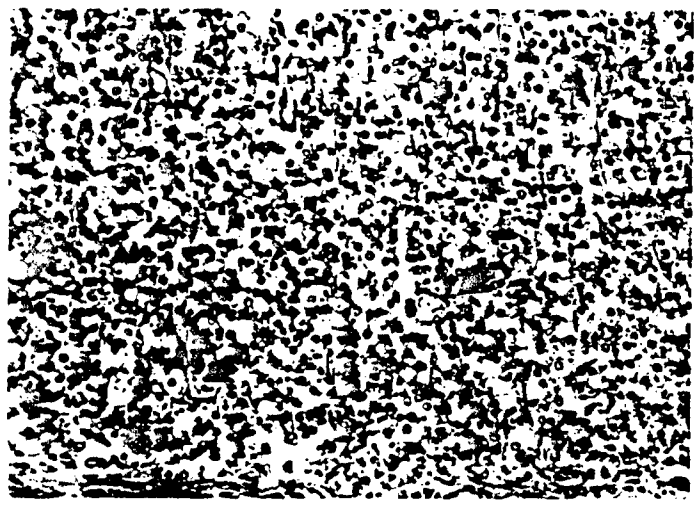


Figure I.7 Spleen from a rat given saline iv at a volume equivalent to the T-2 toxin dose + MPSS ip at 30 mg/kg and killed 14 hr later. Normal splenic architecture is present. (H&E). 62.5 X

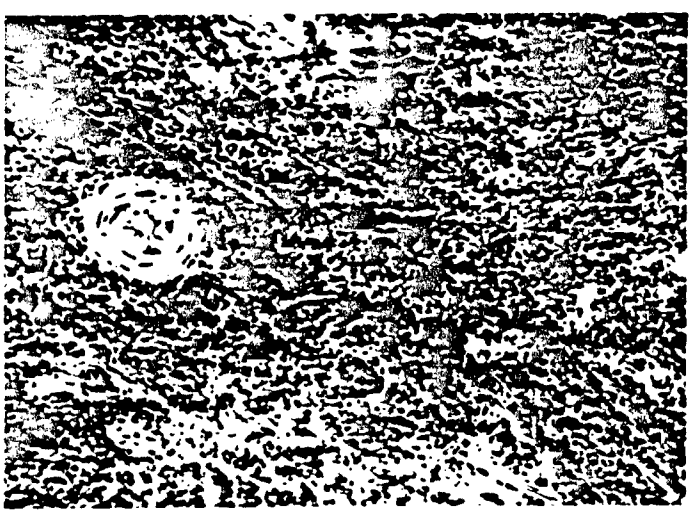
Figure I.8 Spleen from a rat given T-2 toxin iv at 1 mg/kg + MPSS ip at 30 mg/kg and killed 14 hr later. There is widespread lympholysis within the periarteriolar lymphatic sheaths. There is also marked congestion (arrowheads) and cell depletion. (H&E). 62.5 X

Figure I.9 Spleen from a rat given T-2 toxin iv at 1 mg/kg + saline ip at a volume equivalent to the MPSS dose. There is widespread cell necrosis within the periarteriolar lymphatic sheath (arrows). There is also marked congestion. (H&E). 62.5 X

7

8

9

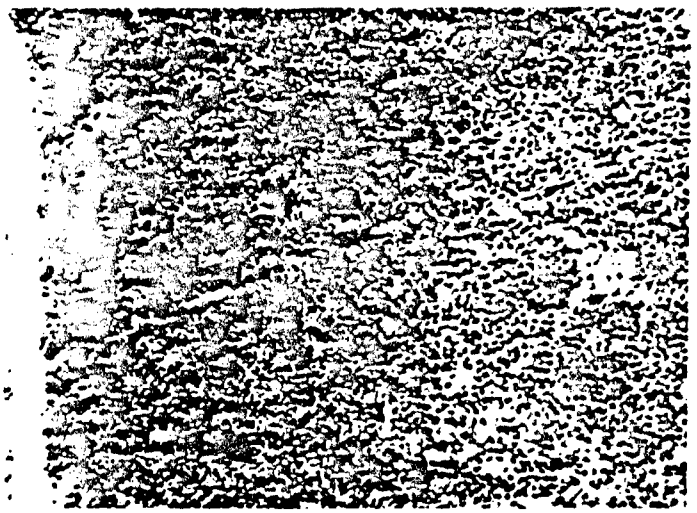


gure I.10 Thymus from a rat given saline iv at a volume equivalent to the T-2 toxin dose + MPSS ip at 30 mg/kg and killed 14 hr later. There is multifocal necrosis of groups of lymphocytes within the cortex (arrows). (H&E). 62.5 X

gure I.11 Thymus from a rat given T-2 toxin iv at 1 mg/kg + MPSS ip at 30 mg/kg and killed 14 hr later. There is multifocal necrosis of groups of lymphocytes within the cortex and medulla (arrows). There is also a marked decrease in cellularity making the demarcation between the cortex and medulla difficult to distinguish. (H&E). 62.5 X

gure I.12 Thymus from a rat given T-2 toxin iv at 1 mg/kg + saline ip at a volume equivalent to the MPSS dose. There is multifocal necrosis of groups of lymphocytes within the cortex (arrows) and medulla. There is also a decrease in cellularity in both the cortex and medulla. (H&E). 62.5 X

10



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12

2. Oral therapy with superactivated charcoal

by
Robert H. Poppenga, Kathy Coddington, Val R. Beasley,
William B. Buck, and Howard Gelberg

Abstract

A semiquantitative scoring system was used to assess changes in the severity of histologic tissue lesions in rats given superactivated charcoal (SAC) po at 1 gram/kg 13 hr and 1 hr before and 6 hr after the administration of T-2 toxin iv at 0.8 mg/kg (an approximate LD_{50} dose). Control rats given T-2 toxin but no SAC developed histologic lesions characteristic of acute T-2 toxicosis in lymphoid tissues such as the thymus, spleen, mesenteric lymph nodes, and Peyer's patches; gastrointestinal tract; and adrenal glands. In rats given T-2 toxin + SAC, there was a marginally significant decrease in the severity of tissue lesions in the duodenum and jejunum ($p = 0.054$ and 0.052, respectively) and a significant decrease in lesion severity in the ileum and closely associated lymphoid tissue (Peyer's patches) ($p = 0.025$ and 0.033, respectively). Therapy had no effect on the severity of lesions in the thymus, spleen, mesenteric lymph nodes, stomach, cecum, pancreas, and adrenal gland.

Introduction

T-2 toxin, a trichothecene mycotoxin, is a secondary fungal metabolite produced primarily by Fusarium species. It has been associated with sporadic outbreaks of human and animal disease in several regions of the world (Hsu et al., 1972; Ueno et al., 1972; Puls and Greenway, 1976; Yagen and Joffe, 1976). T-2 toxin has also been implicated as a component of the chemical warfare agent "yellow rain" allegedly used in Southeast Asia and Afghanistan (Mirocha et al., 1983; Rosen and Rosen, 1982).

The pathophysiology of acute T-2 toxicosis has been studied in a variety of experimental mammalian species (Sato et al., 1975; DeNicola et al., 1978; Weaver et al., 1978; Feuerstein et al., 1985; Lorenzana et al., 1985). Administration of a lethal dose of the toxin causes circulatory shock characterized by declines in cardiac output and systemic arterial blood pressure, alterations in organ blood flow, and lactic acidosis (Beasley et al., 1987; Lorenzana et al., 1985; Feuerstein et al., 1985; Lundeen et al., 1986; Siren and Feuerstein, 1986). Morphologic changes include: severe necrosis of lymphoid tissues; severe congestion and necrosis of the gastrointestinal mucosa; myocardial degeneration and necrosis with mineralization; pancreatic degeneration and necrosis; and adrenal gland parenchymal cell necrosis (DeNicola et al., 1978; Brennecke and Nuefeld, 1982; Pang et al., 1985 and 1987; Thurman et al., 1986).

The most successful therapeutic measures for treating the acute toxicosis include iv administration of high doses of water-soluble glucocorticosteroids (Fricke, 1985; Tremel et al., 1985; Poppenga et al., 1987a), glucocorticosteroids in combination with other supportive care (Poppenga et al., 1987b), and oral administration of the intestinal adsorbents, SAC and cholestryamine (Galey et al., 1987; Poppenga et al., 1987b; Fricke and Jorge, 1986).

The objectives of this report are: 1) to describe the effect of orally administered SAC on rats given an acutely toxic dose of T-2 toxin iv with regard to the severity of toxin-induced microscopic lesions in target organs and 2) to discuss significant findings as they may relate to the efficacy of SAC as a treatment for the acute mycotoxicosis. The efficacy of orally administered SAC in the prevention of toxin-induced death in these rats has been reported elsewhere (see Section I.B).

Materials and Methods

Animals

Approximately 225 g, female, Harlan Sprague-Dawley rats were allowed to acclimate to their housing for a minimum of 1 week prior to inclusion in the study. They were fed a standard ration which contained no detectable concentrations of the trichothecene mycotoxins, T-2 toxin, diacetoxyscirpenol, or deoxynivalenol (detection limit 0.5 ppm); zearalenone (detection limit 0.5 ppm); or aflatoxin (detection limit 10 ppb).

Toxin and Drug Agent

Purified T-2 toxin was obtained from the Toxicology Analytical Laboratory, University of Illinois, College of Veterinary Medicine. Toxin purity was $\geq$ 95% as assessed by GC using a FID detector. The toxin was dissolved in 50% ethanol:50% saline to a concentration of 1 mg/ml.

SAC (SuperChar®) was supplied courtesy of Gulf Biosystems Inc., Dallas, TX. The SAC was suspended in normal saline to a concentration of 100 mg (dry weight) per ml.

Experimental Protocol

Rats were randomly assigned to 1 of 3 treatment groups using a random number table (Sokol and Rohlf, 1973) as follows. Group 1 (n = 12) was given normal saline via gavage, at a volume equivalent to the SAC slurry, 13 hr and 1 hr prior to, and 6 hr after the T-2 toxin vehicle (50% ethanol:50% normal saline) given iv at a volume equivalent to the T-2 toxin dose. Group 2 (n = 15) was given normal saline (0.9%) via gavage at a volume equivalent to the SAC slurry 13 hr and 1 hr prior to and 6 hr after T-2 toxin given iv at 0.8 mg/kg. Group 3 (n = 15) was given SAC via gavage at 1 gm (dry weight) per kg in a sluury 13 hr and 1 hr prior to and 6 hr after T-2 toxin given iv at 0.8 mg/kg. After dosing, the rats were offered food and water *ad libitum*.

Rats dying spontaneously during a 72 hr observation period were immediately necropsied and tissues placed in 10% neutral buffered formalin. Animals surviving for 72 hr were anesthetized with methoxyflurane, exsanguinated, and necropsied. Tissue sections from the stomach, small intestine, cecum, thymus, spleen, mesenteric lymph nodes, adrenal, and pancreas were embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin, and examined by light microscopy.

To determine whether there was a treatment effect on the severity of T-2 toxin-induced lesions, a quantitative evaluation of the microscopic changes present was performed using a semiquantitative scoring system modified from Coppock et al. (1985). The total lesion score for intestinal tract-associated lymphoid tissue (Peyer's patches) and

mesenteric lymph nodes was the product of the number of follicular areas involved and severity of lymphoid necrosis within germinal centers. The number of follicular areas involved was scored from 0 to 3 as follows: 0 = normal background, 1 = occasional, 2 = most, and 3 = all. The severity of lymphoid necrosis as a percentage of lymphoid cells which were necrotic was scored from 0 to 4 as follows: 0 = normal background, 1 = $\leq$ 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%. The possible scores for both lymphoid tissues ranged from 0 to 12. The total lesion score for the thymus was the sum of the scores for severity of lymphoid necrosis in the cortex and medulla, respectively, with the severity of lymphoid necrosis scored as above. The possible scores ranged from 0 to 8. The score for splenic lesions was the sum of the products of the estimated number of follicles involved (B-cell areas) x the score for percent lympholysis and the estimated number of periarteriolar cuffs involved (T-cell areas) x the score for percent lympholysis. The estimated number of splenic follicles or periarteriolar cuffs involved was scored as 0 = normal background, 1 = occasional, 2 = most, and 3 = all. Percent lymphoid necrosis was scored as above. The possible scores ranged from 0 to 24.

The total lesion score for the stomach was the sum of the degree of congestion/hemorrhage and degree of necrosis scored as 0 = normal background, 1 = mild, 2 = moderate, and 3 = severe. The possible scores ranged from 0 to 6. The total lesion scores for the duodenum, jejunum, ileum, and cecum were the product of the percentage of crypts affected and the percent epithelial cell necrosis within individual crypts with each scored from 0 to 4 as follows: 0 = normal background, 1 = 10 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%. The possible scores ranged from 0 to 16.

The total lesion score for the adrenal was the sum of the degree of congestion/hemorrhage and degree of necrosis and inflammatory cell infiltration scored as 0 = normal background, 1 = slight, 2 = mild, 3 = moderate, and 4 = severe. The possible scores ranged from 0 to 8. The total lesion score for the pancreas was the product of the number of acini involved scored as 1 = normal background, 2 = occasional, and 3 = many and the number of necrotic cells within each acinus scored as 1 = normal background, 2 = up to 1/3, 3 = 1/3 to 2/3, and 4 $\geq$ 2/3. The possible scores ranged from 0 to 12.

Statistical Analysis

Two investigators separately scored the histologic lesions for each tissue according to the above grading criteria. Tissues in the negative control group given toxin vehicle + saline gavage (Group 1) were examined but not included in the statistical evaluation. For the treatment groups given T-2 toxin + saline (Group 2) and T-2 toxin + SAC (Group 3), the 2 sets of ranks were pooled according to the treatment. Differences between Groups 2 and 3 for each tissue were then evaluated by the Kruskal-Wallis 1-way analysis of variance by ranks test (Kruskal and Wallis, 1952) using a computer statistical package (Systat Version 3.2, Systat, Inc., Evanston, IL). A probability level equal to or less than 5% ($p \leq 0.05$) was considered significant.

Results

In the positive control group (Group 2), histologic lesions in the tissues examined were characteristic of acute T-2 toxicosis. In the Peyer's patches and mesenteric lymph nodes, severe necrosis and depletion of lymphocytes within all germinal centers were seen. Histologic lesions in the spleen were characterized by severe congestion and cell depletion in both B- and T-cell regions. Marked lympholysis occurred in splenic follicular germinal centers. In the thymus, marked lympholysis occurred in the cortex with less severe lympholysis noted in the medulla.

The gastric lesions were restricted to the glandular region and were characterized as a necrotizing, hemorrhagic gastritis. In general, necrosis predominated in the lower half of the mucosa with hemorrhage more evident in the upper half. It was difficult to determine whether parietal or chief cells were more susceptible to damage. Enteric lesions consisting of pyknosis, karyorrhexis, and necrosis of crypt enterocytes occurred. The basal portions of the crypts were often filled with cellular debris and the lamina propria contained numerous inflammatory cells. The lesions were qualitatively less severe in the cecum compared to the duodenum, jejunum, and ileum.

The pancreas was relatively unaffected although individual acinar cell degeneration and necrosis occurred. The islet cells were unaffected. The zona fasiculata of the adrenal gland had areas of congestion and hemorrhage; inflammatory cell infiltration; and small, multifocal areas of parenchymal cell necrosis. In addition, inflammatory cell infiltration was noted in the zona glomerulosa.

The raw scores for lesion severity for each tissue examined and their corresponding ranks are given in Table I.7. Marginally significant differences in lesion severity between the group given T-2 toxin + saline and the group given T-2 toxin + SAS occurred for the duodenum and jejunum ($p = 0.054$ and 0.052, respectively). There were significant differences for the ileum and Peyer's patches ($p = 0.025$ and 0.033, respectively). No significant decrease in lesion severity was noted for the thymus, spleen, mesenteric lymph nodes, glandular portion of the stomach, cecum, pancreas, or adrenal gland.

Figs. I.14 through I.16 illustrate differences in the histologic appearance of the duodenum between rats in Groups 1, 3, and 2, respectively. Figs. I.17 through I.19 illustrate differences in the histologic appearance of the ileum between rats in Groups 1, 3, and 2, respectively. Figs. I.20 through I.22 illustrate representative histologic changes in the spleen from rats in the various groups. Fig. I.20 is from a negative control rat (Group 1). There is slight lymphoid necrosis within the germinal center of the splenic follicle. This was considered a normal background degree of lympholysis. As noted from Table I.7, scores for the severity of splenic lesions were either zero or maximal (24). Fig. I.21 illustrates a spleen given a score of 0, while Fig. I.22 illustrates a spleen given a maximal score. There were no differences in overall scores for lesion severity within the spleen between Groups 2 (T-2 toxin + SAC) and 3 (T-2 toxin + saline).

Discussion

There is evidence both in vitro and in vivo that T-2 toxin is rapidly metabolized by the liver and eliminated via the bile, primarily as glucuronide conjugates (Pace et al., 1985; Corley et al., 1985; Gareis et al., 1986). There is also evidence suggesting that intestinal microflora may deconjugate these glucuronide metabolites (Corley et al., 1985) which, in turn, are likely to be rapidly reabsorbed from the intestinal tract. If this is the case, it is possible that continued enterohepatic recirculation of toxic metabolites prolongs the exposure of intestinal epithelial cells to the effects of the toxin and contributes to cell death. In addition, reabsorption of toxic metabolites into the systemic circulation may exacerbate the acute toxicosis.

In vitro, several intestinal adsorbents effectively bind T-2 toxin (Fricke and Poppenga, 1987; Bratich and Buck, 1987). Superactivated charcoal (SAC), a high surface area form of activated charcoal, is particularly effective. In vivo, SAC is efficacious for preventing mortality in rodents and swine following the oral administration of otherwise lethal doses of T-2 toxin (Galey et al., 1987; Coddington, 1986). A significant decrease in mortality was demonstrated even when the administration of the charcoal was delayed for up to 3 hr after exposure (Galey et al., 1987). Of particular interest is the observation that the oral administration of SAC was efficacious for treating acute toxicosis following sc or iv exposure to the toxin (Fricke and Poppenga, 1987; Poppenga et al., 1987b).

The results of the present study suggest that SAC may have exerted its beneficial action on intestinal lesion severity and rat survival in 2 ways. First, SAC may have adsorbed toxic metabolites within the intestinal tract following their formation in the liver and elimination via the bile. Adsorption could include metabolites eliminated in a free, unconjugated form or those eliminated as glucuronide conjugates and subsequently deconjugated by intestinal microflora. This would effectively prevent a direct cellular action of the metabolites on intestinal epithelial cells and nearby lymphoid cells in Peyer's patches. The decreased exposure of intestinal epithelial cells to metabolites of T-2 toxin may contribute to improved cell viability and minimize secondary effects resulting from cell death such as impairment of local microcirculation. Microcirculatory impairment could lead to mucosal ischemia and hypoxia, subsequent cell damage, and the release of cardioinhibitory factors. Such factors have been demonstrated in the blood of cats in a small intestinal regional shock model (Lundgren and Haglund, 1978). It is also conceivable that the SAC could bind conjugated metabolites and prevent their deconjugation, although, to the authors' knowledge, the binding affinity of charcoal for conjugates has not been investigated.

A second mode of beneficial action, related to the above hypothesis, would be the prevention of systemic exposure to toxic metabolites as a result of enterohepatic recirculation. T-2 toxin, given in sufficient doses, is known to cause marked declines in aortic mean pressure and severe alterations of organ blood flow in experimental animals acutely exposed to the toxin (Lorenzana et al., 1985; Lundeen, et al., 1986; Siren and Feuerstein, 1986; Beasley et al., 1987). In swine given acutely toxic doses of T-2 toxin iv, blood flow to the small intestine was initially increased with a subsequent decline approximately 4.5 hr

after dosing (Beasley et al., 1987). Mucosal lesions in the small intestine have been reported to occur in other circulatory shock states, especially endotoxic shock (Haglund et al., 1984). The occurrence of the lesions has been correlated with low systemic arterial blood pressure in the absence of a reduction in small intestinal blood flow (Falk et al., 1985). Despite the normal small intestinal blood flow, Falk et al. (1985) hypothesized that the intestinal lesions were due to mucosal ischemia and hypoxia. Initial hemodynamic alterations in acute T-2 toxicosis closely mimic those noted in endotoxic shock. The intestinal lesions in acute T-2 toxicosis and endotoxic shock may therefore have a common pathogenesis due in part to the similar systemic effects of T-2 toxin and endotoxin. The prevention of systemic absorption of deconjugated, toxic metabolites by SAC could conceivably lower the systemic exposure to these compounds and prevent or ameliorate hemodynamic alterations which probably lead to small intestinal mucosal ischemia and hypoxia.

It is also quite possible that SAC exerts its beneficial action in both ways. Early in the toxicosis, SAC may prevent direct cytotoxic actions on intestinal epithelial cells. Later, due to lowered systemic exposure to T-2 toxin, SAC may prevent mucosal damage secondary to systemic hemodynamic alterations. Regardless of which hypothesis, if either is correct, reduced severity of mucosal lesions may prevent the entrance into a vicious cycle in which mucosal damage results in the release of toxic amounts of cardioinhibitory factors, leading to exacerbation of hypotension and causing more mucosal damage.

In summary, the present study may contribute to our understanding of the way in which SAC given orally lessens mortality following parenteral exposure to T-2 toxin. It may also explain why SAC is efficacious when given several hr after oral exposure to the toxin; long after an orally administered dose of T-2 toxin would be absorbed from the gastrointestinal tract.

Acknowledgements

The authors are grateful to Tina Kerferlis and Mary Busse for their excellent technical help. Our thanks also to Laura Beachy for typing the manuscript and table.

These studies were supported in part by the U.S. Army Medical Research and Develpoment Command, Contract No. DAMD 17-82-C-2179 and 17-85-C-5224. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

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Table I.7 Superactivated charcoal study. Raw scores and corresponding ranks of lesions from tissues examined histologically.

Treatment Group	Tissue: Thymus 1		Thymus 2		Spleen 1		Spleen 2		Mesenteric Lymph Node 1		Mesenteric Lymph Node 2		Stomach 1		Stomach 2		Duodenum 1		Duodenum 2		Jejunum 1		Jejunum 2	
T-2 Toxin + Saline																								
Animal ID	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R
1A	2	17.50	4	36.50	0	10.50	0	10.50	2	16.50	4	20.50	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
3	5	43.00	8	56.00	24	40.00	24	40.00	12	46.00	12	46.00	6	30.00	N/A		1	28.50	1	28.50	1	25.00	2	29.00
6A	2	17.50	3	27.50	24	40.00	24	40.00	6	24.50	12	46.00	2	21.50	N/A		12	43.00	12	43.00	4	31.50	16	53.00
8	3	27.50	3	27.50	18	23.00	24	40.00	12	46.00	12	46.00	2	21.50	N/A		16	53.00	16	53.00	16	53.00	16	53.00
12	2	17.50	3	27.50	24	40.00	24	40.00	9	30.50	12	46.00	5	28.50	N/A		8	36.50	16	53.00	12	42.50	12	42.50
18A	3	27.50	1	8.00	0	10.50	0	10.50	1	13.50	0	6.00	0	2.00	N/A		0	13.00	0	13.00	0	11.50	0	11.50
20	7	52.00	8	56.00	24	40.00	24	40.00	12	46.00	9	30.50	2	21.50	N/A		12	43.00	16	53.00	12	42.50	16	53.00
21	5	43.00	5	43.00	24	40.00	24	40.00	12	46.00	12	46.00	4	27.00	N/A		16	53.00	16	53.00	16	53.00	16	53.00
18	3	27.50	6	48.00	24	40.00	24	40.00	12	46.00	9	30.50	1	10.50	N/A		12	43.00	16	53.00	12	42.50	16	53.00
6B	1	8.00	0	2.00	1	21.00	0	10.50	3	18.00	0	6.00	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
7	2	17.50	1	8.00	21	25.00	24	40.00	4	20.50	6	24.50	2	21.50	N/A		12	43.00	16	53.00	12	42.50	16	53.00
11	2	17.50	2	17.50	24	40.00	24	40.00	12	46.00	12	46.00	2	21.50	N/A		N/A	---	N/A	---	N/A	---	N/A	---
14	2	17.50	4	36.50	24	40.00	24	40.00	9	30.50	8	27.00	2	21.50	N/A		12	43.00	16	53.00	8	36.00	16	53.00
17	8	56.00	4	36.50	24	40.00	24	40.00	12	46.00	12	46.00	1	10.50	N/A		12	43.00	16	53.00	12	42.50	16	53.00
18B	8	56.00	3	27.50	N/A	---	N/A	---	N/A	---	N/A	---	2	21.50	N/A		0	13.00	0	13.00	4	31.50	1	25.00
		445.50		458.00		450.00		471.50		476.00		467.00		280				481.00		547.5		477.00		555.00
T-2 Toxin + S.A.C.																								
4	5	43.00	8	56.00	24	40.00	24	40.00	12	46.00	12	46.00	1	10.50	N/A		0	13.00	1	28.50	1	25.00	1	25.00
5	3	27.50	2	17.50	0	10.50	0	10.50	1	13.50	0	6.00	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
7	N/A	---	N/A	---	0	10.50	0	10.50	0	6.00	0	6.00	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
13	6	48.00	4	36.50	24	40.00	24	40.00	12	46.00	12	46.00	1	10.50	N/A		8	36.50	6	33.00	8	36.00	12	42.50
14	7	52.00	6	48.00	24	40.00	24	40.00	12	46.00	12	46.00	1	10.50	N/A		1	28.50	6	33.00	2	29.00	6	33.50
16A	3	27.50	0	2.00	0	10.50	0	10.50	1	13.50	0	6.00	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
17	1	8.00	0	2.00	0	10.50	0	10.50	0	6.00	0	6.00	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
22	2	17.50	5	43.00	20	24.00	24	40.00	12	46.00	6	24.50	5	28.50	N/A		8	36.50	16	53.00	6	33.50	12	42.50
2	3	27.50	1	8.00	2	22.00	0	10.50	0	6.00	0	6.00	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
9	2	17.50	1	8.00	0	10.50	0	10.50	1	13.50	0	6.00	0	2.00	N/A		0	13.00	0	13.00	0	11.50	0	11.50
12	1	8.00	4	36.50	0	10.50	0	10.50	2	16.50	4	20.50	0	2.00	N/A		0	13.00	0	13.00	0	11.50	0	11.50
15	4	36.50	6	48.00	24	40.00	24	40.00	12	46.00	12	46.00	3	26.00	N/A		8	36.50	6	53.00	8	36.00	12	42.50
16B	1	8.00	1	8.00	0	10.50	0	10.50	6	24.50	4	20.50	1	10.50	N/A		0	13.00	0	13.00	0	11.50	0	11.50
19	4	36.50	6	48.00	N/A	---	N/A	---	12	46.00	9	30.50	1	10.50	N/A		12	43.00	12	43.00	12	42.50	16	53.00
20	7	52.00	4	36.50	N/A	---	N/A	---	12	46.00	9	30.50	2	21.50	N/A		1	28.50	1	28.50	1	25.00	1	25.00
		409.50		398.00		279.50		284.00		421.50		346.50		197.50				326.50		376.00		319.00		356.00
		p = 0.947				p = 0.104				p = 0.191				p = 0.113				p = 0.054				p = 0.052		

Table 1.7 continued

Treatment Group	Ileum 1		Ileum 2		Cecum 1		Cecum 2		Pancreas 2		Pancreas 2		Adrenal 1		Adrenal 2		Peyer's Patches 1		Peyer's Patches 2	
T-2 Toxin + Saline																				
Animal ID	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R	RS	R
1A	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	0	7.00	1	15.50	3	9.00	6	16.50
3	1	24.00	2	26.50	1	30.00	1	30.00	1	48.00	0	20.50	3	29.50	4	37.50	12	41.00	9	23.00
6A	12	39.50	16	51.50	1	30.00	1	30.00	1	48.00	4	60.00	6	53.00	5	46.00	12	41.00	12	41.00
8	16	51.50	16	51.50	4	45.50	8	57.00	1	48.00	0	20.50	3	29.50	3	29.50	12	41.00	12	41.00
12	12	39.50	16	51.50	2	38.50	0	12.00	1	48.00	0	20.50	5	46.00	3	29.50	12	41.00	12	41.00
18A	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	0	7.00	0	7.00	4	12.00	0	4.50
20	16	51.50	16	51.50	1	30.00	1	30.00	1	48.00	0	20.50	4	37.50	2	21.00	12	41.00	12	41.00
21	12	39.50	16	51.50	4	45.50	6	52.50	1	48.00	0	20.50	3	29.50	2	21.00	12	41.00	12	41.00
18	12	39.50	16	51.50	8	57.00	6	52.50	0	20.50	0	20.50	5	46.00	5	46.00	12	41.00	12	41.00
6B	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	0	7.00	0	7.00	N/A	---	N/A	---
7	12	39.50	16	51.50	4	45.50	4	45.50	0	20.50	0	20.50	N/A	---	N/A	---	12	41.00	12	41.00
11	N/A	---	N/A	---	N/A	---	N/A	---	2	57.50	0	20.50	7	55.00	5	46.00	12	41.00	12	41.00
14	4	31.00	12	39.50	4	45.50	6	52.50	1	48.00	0	20.50	8	56.00	5	46.00	12	41.00	12	41.00
17	12	39.50	16	51.50	4	45.50	3	41.00	2	57.50	1	48.00	3	29.50	3	29.50	12	41.00	12	41.00
18B	4	31.00	4	31.00	1	30.00	0	12.00	2	57.50	1	48.00	4	37.50	5	46.00	12	41.00	12	41.00
		560.50		543.50		479.00		451.00		610.70		523.50		470.00		427.50		513.00		495.00
T-2 Toxin + S.A.C.																				
4	2	26.50	4	31.00	1	30.00	1	30.00	1	48.00	0	20.50	2	21.00	2	21.00	8	20.50	12	41.00
5	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	1	15.50	1	15.50	0	4.50	0	4.50
7	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	0	7.00	0	7.00	0	4.50	0	4.50
13	4	31.00	16	51.50	2	38.50	2	38.50	1	48.00	0	20.50	4	37.50	3	29.50	8	20.50		41.00
14	1	24.00	4	31.00	1	30.00	2	38.50	1	48.00	0	20.50	3	29.50	3	29.50	8	20.50	12	41.00
16A	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	1	15.50	0	7.00	12	41.00	6	16.50
17	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	0	7.00	0	7.00	4	12.00	0	4.50
22	12	39.50	16	51.50	8	57.00	6	52.50	1	48.00	0	20.50	6	53.00	5	46.00	12	41.00	12	41.00
2	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	2	21.00	0	7.00	8	20.50	0	4.50
9	0	11.50	0	11.50	1	30.00	0	12.00	0	20.50	0	20.50	N/A	---	N/A	---	4	12.00	0	4.50
12	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	2	21.00	2	21.00	6	16.50	6	16.50
15	12	39.50	16	51.50	4	45.50	6	52.50	1	48.00	0	20.50	5	46.00	4	37.50	12	41.00	12	41.00
16B	0	11.50	0	11.50	0	12.00	0	12.00	0	20.50	0	20.50	0	7.00	0	7.00	4	12.00	4	12.00
19	12	39.50	16	51.50	4	45.50	6	52.50	1	48.00	0	20.50	6	53.00	5	46.00	12	41.00	12	41.00
20	1	24.00	4	31.00	1	30.00	1	30.00	0	20.50	0	20.50	5	46.00	4	37.50	12	41.00	12	41.00
		316.00		391.00		390.50		390.50		472.00		307.50		380.00		318.50		348.50		354.50
		p = 0.025				p = 0.285				p = 0.082				p = 0.322				p = 0.033		

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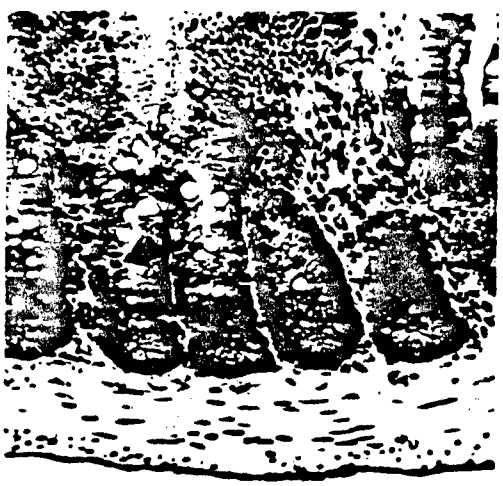
ure I.14 Duodenum from a rat given normal saline via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin vehicle (50% ethanol:50% saline) iv. Note the normal crypt architecture and the presence of mitotic activity within the crypts (arrow). (H&E). 62.5 X

ure I.15 Duodenum from a rat given SAC via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin iv at 0.8 mg/kg. Note the normal crypt architecture and the presence of mitotic activity within the crypts (arrow). (H&E). 62.5 X

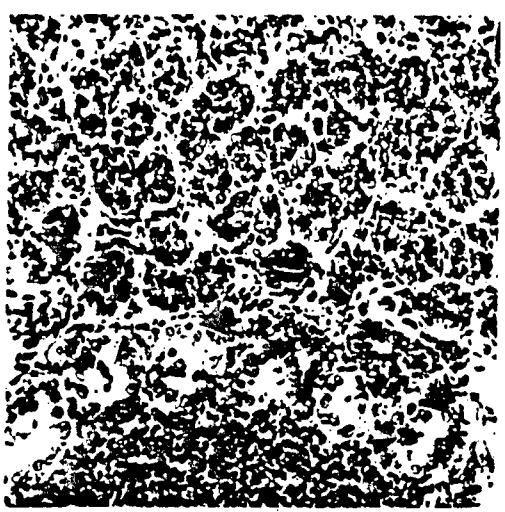
ure I.16 Duodenum from a rat given saline via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin iv at 0.8 mg/kg. Note the widespread crypt epithelial cell necrosis (arrows) and debris with the crypt lumen (arrowhead). (H&E). 62.5 X

14

15



16



ıre I.17 Ileum from a rat given normal saline via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin vehicle (50% ethanol:50% saline) iv. Note the normal crypt architecture and the presence of mitotic activity within the crypts (arrows). (H&E). 62.5 X

ıre I.18 Ileum from a rat given SAC via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin iv at 0.8 mg/kg. Note the normal crypt architecture and the presence of mitotic activity within the crypts (arrows). (H&E). 62.5 X

ıre I.19 Ileum from a rat given saline via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin iv at 0.8 mg/kg. There is widespread necrosis of crypt epithelial cells. Note the debris within the crypt lumen (arrows). (H&E). 62.5 X

17

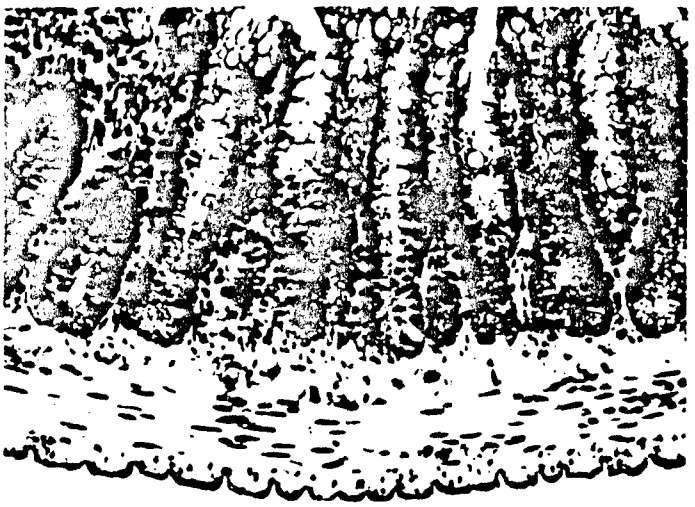


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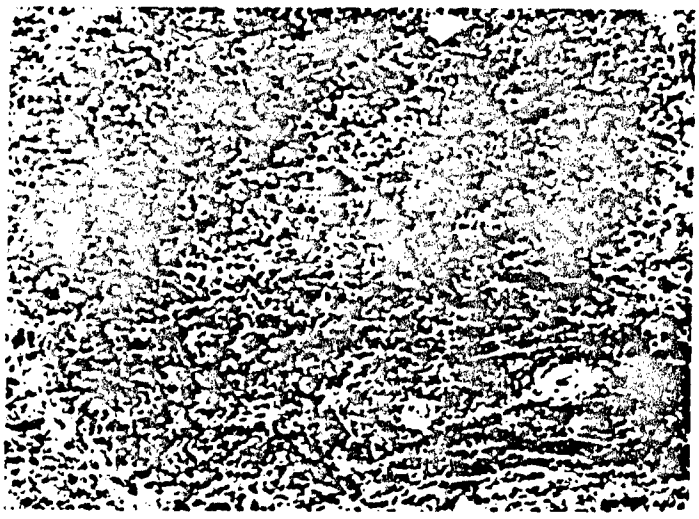
Figure I.20 Spleen from a rat given normal saline via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin vehicle (50% ethanol:50% saline) iv. There is slight lympholysis within the germinal center of the splenic follicle (arrow). This was considered to be a normal background level of necrosis. No other histologic lesions were noted. (H&E). 62.5 X

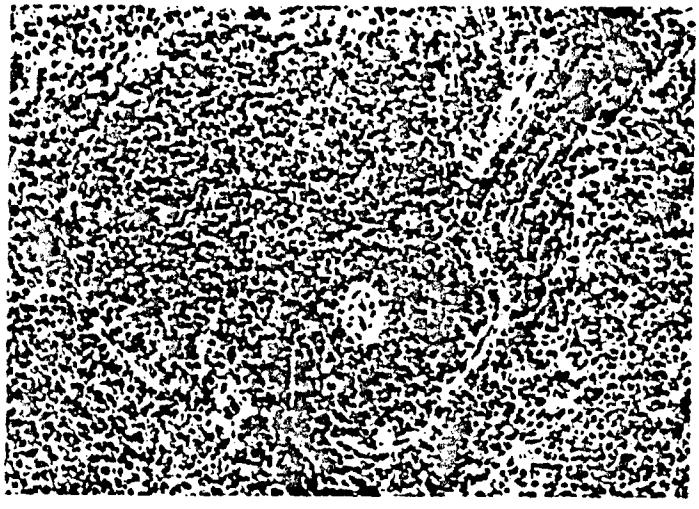
Figure I.21 Spleen from a rat given SAC via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin iv at 0.8 mg/kg. This is representative of a spleen given a zero score. (H&E). 62.5 X

Figure I.22 Spleen from a rat given saline via gavage 13 hr and 1 hr prior to and 6 hr after T-2 toxin iv at 0.8 mg/kg. This is representative of a spleen given a maximal score (24). There is marked necrosis of lymphocytes within the periarteriolar sheath (arrows). There is also widespread, severe congestion (arrowheads). There were no differences detected in the severity of splenic lesions between rats in Group 2 (T-2 toxin + SAC) and rats in Group 3 (T-2 toxin + saline). (H&E). 62.5 X

20



21



22

D. Assessment of Ascorbic Acid and Dexamethasone in Combination with PGE_1 for the Treatment of Acute T-2 Toxicosis in the Rat

by
Robert H. Poppenga, Barbara L. Kindler, Val R. Beasley,
David J. Schaeffer, and William B. Buck

Summary

Ascorbic acid (AA) was given ip to female Sprague Dawley rats 12 hr prior to and/or immediately after the iv administration of a dose of T-2 toxin approximately 1.5X the LD_{50} to assess its efficacy as a treatment for acute T-2 toxicosis. In addition, the slow iv infusion of prostaglandin E_1 (PGE_1) over a 2 hr period at 50 µg/kg or a combination therapy, consisting of an iv bolus of dexamethasone sodium phosphate (DEX) at 6 mg/kg and a slow iv infusion of PGE_1 at 5, 25, or 50 µg/kg, was evaluated as a treatment for acute toxicosis in the rat. Ascorbic acid did not improve survival at any dose evaluated and, in fact, had a detrimental effect on median survival times when given ip either immediately after or 12 hr before and immediately after the administration of T-2 toxin. PGE_1 given alone at 50 µg/kg by slow iv infusion did not improve survival. The combination of DEX and PGE_1 did improve survival times compared with the control group of animals given T-2 toxin and no therapy. However, survival with the combination therapy was no better and, in the case of the groups given PGE_1 at either 25 or 50 µg/kg, was apparently worse than that obtained with DEX alone.

Introduction

T-2 toxin [3 alpha-hydroxy-4 beta, 15-diacetoxy-8 alpha (3-methyl-butyryloxy)-12,13-epoxytrichothec-9-ene] is produced by various species of Fusarium. It belongs to a large group of mycotoxins, the trichothecenes, which have antifungal, antibacterial, antiviral, phytotoxic, and cytotoxic properties.[1]

In experimental animals given single, high doses of T-2 toxin by the oral, iv, or inhalation routes, a number of organ systems are affected, especially those with rapidly dividing cells such as the lymphoid tissues, gastrointestinal tract and bone marrow.[2-4] Acute exposure to sufficient T-2 toxin results in the rapid onset of circulatory shock characterized by reduced cardiac output, profound arterial hypotension, lactic acidosis, declines in blood flow to vital organs, and death within 72 hr.[5-8]

The mechanism(s) of action responsible for the clinical manifestations of acute T-2 toxicosis are not clear. In vitro and in vivo, T-2 toxin is a potent inhibitor of eukaryotic protein and DNA synthesis.[9-11] T-2 toxin decreased liver concentrations of glutathione in mice[12] and there is evidence in vitro that high levels of the toxin can alter membrane function and structure.[13,14]

Therapeutic approaches for the treatment of acute T-2 toxicosis have focused on agents effective in other circulatory shock states or those which might attenuate hypothesized mechanisms of action of the toxin. Administration of water-soluble salts of the glucocorticosteroids dexamethasone (DEX) and methylprednisolone improved survival times and rates in mice and rats when given shortly after administration of an otherwise lethal dose of the toxin.[15-17] Antioxidants such as ascorbic acid (AA),[18] the glutathione prodrugs L-2-oxo- and L-2-methyl-derivatives of thiazolidine-4-carboxylate,[19] and vitamin E[a] decreased the lethal toxicity of T-2 toxin in mice. However,

AA did not improve the survival rate of rats given T-2 toxin.[17] Gastrointestinal adsorbents such as superactivated charcoal and cholestyramine have also improved survival in mice, rats, and swine following oral or parenteral exposure to T-2 toxin.[20-22] Administration of PGE_1 has improved survival of animals in both endotoxic[23] and hemorrhagic[24] shock. In addition, the combination of PGE_1 and a glucocorticosteroid (methylprednisolone sodium succinate) improved the survival rate of Sprague Dawley rats given endotoxin over that obtained with either agent alone.[b]

One goal of our study was to more thoroughly evaluate AA for the treatment of acute T-2 toxicosis in rats. Another goal was to assess whether PGE_1 either alone or in combination with a water-soluble salt of DEX would improve survival of rats over that obtained with DEX alone.

Materials and Methods

Ascorbic Acid Treatment Study

Following a minimum acclimation period of 7 days, approximately 225 gram, female Harlan Sprague Dawley rats[c] were randomly assigned to 1 of 5 treatment groups (Table I.8). Twenty-four hr prior to the study, the rats were weighed and appropriate doses of T-2 toxin[d] and ascorbic acid[e] were prepared. Food but not water was witheld for 12 hr prior to toxin administration. All animals except those in the T-2 toxin positive control and T-2 toxin vehicle + AA control groups (Groups 1 and 2, respectively) were then given a bolus injection of T-2 toxin at 1 mg/kg (an approximate 1.5X LD_{50} dose) followed immediately by AA given ip at 400, 800, or 1200 mg/kg. Group 1 controls were given a bolus injection of T-2 toxin iv at 1 mg/kg followed by normal saline ip in a volume equivalent to the AA doses. Group 2 controls were given a bolus injection of the toxin vehicle (50% ethanol:50% saline) via the tail vein followed by AA given ip at 1200 mg/kg.

After dosing, all animals were returned to their cages and periodically observed. Feed and water were available ad libitum. Survival times were recorded for rats that died spontaneously. Animals surviving 72 hr were anesthetized with methoxyflurane[f] and exsanguinated. Since the true survival time of animals living the full 72 hr observation period was not determined, these data were considered to be temporally censored. Treatment groups and dosing protocols are given in Table I.8.

Ascorbic Acid Pretreatment Study

Identical procedures as in the first AA study were followed except the AA dosing regimen was modified. In this study, all the rats were pretreated ip with equivalent volumes of either saline or AA given at 400, 800, or 1200 mg/kg 12 hr before the administration of T-2 toxin iv at 1 mg/kg. Immediately following the administration of the toxin, the rats were given a second ip injection of either saline or AA in an amount identical to the first dose. As before, all animals were returned to their cages and periodically observed. Treatment groups and dosing protocols are given in Table I.9.

Dexamethasone + PGE_1 Study

Rats were randomly assigned to 1 of 6 treatment groups (Table I.10). Food but not water was witheld for 12 hr prior to treatment. Approximately 1 hr before administration of T-2 toxin, each animal was anesthetized with

methoxyflurane and a 26 gauge, 3/4 inch indwelling catheter[g] was placed in a tail vein and secured with adhesive tape. The catheter was flushed with sterile heparinized saline.[h] The animals were next placed in individual stainless steel restraining devices and allowed to recover from anesthesia.

T-2 toxin was given iv at 1 mg/kg via the catheter followed immediately by dexamethasone sodium phosphate[i] at 6 mg/kg using the same catheter. The catheter was flushed with sterile heparinized saline before and after each agent. An infusion of PGE_1[j] was started immediately after the DEX administration. The concentration of PGE_1 varied depending on the treatment group so that the total calculated dose of PGE_1 was infused at a slow, constant rate via the catheter over 2 hr. Each animal was similarly administered a second, equivalent dose of DEX iv 4 hr after the first dose. After the time of the second DEX injection, all rats were removed from the restraining devices and returned to their cages. Food and water were available ad libitum.

As in previous studies, the rats were observed for 72 hr. Animals surviving the full 72 hr observation period were anesthetized with metofane and exsanguinated.

Statistical Analysis

A k-sample test capable of handling $k > 2$ with censored observations was used to detect overall significant differences in the survival curves for the treatment groups within each study.[25] Since only improved survival was hypothesized, a 1-tailed test of significance was used ($p \leq 0.025$). If overall significance was found, the proportion of rats surviving in each experimental group was calculated for arbitrarily selected time intervals for the 72 hr observation period. The proportions were normalized using arcsin transformation and means for each treatment group were determined using the transformed data. Dunnett's multiple comparison test for comparing several treatments with a control[26] was used to determine differences between the individual treatment groups and the positive control group. For the DEX + PGE_1 study, possible therapeutic synergism between the 2 drugs was evaluated using a modified bootstrap procedure.[27]

The proportion of animals surviving over time was calculated for each treatment group using the product-limit method of Kaplan and Meier.[28] Survival curves for the AA and the DEX + PGE_1 studies are presented in Figs. I.23 and I.24, respectively.

Results

As can be seen in Table I.8 and Fig. I.23a, there was no improvement in survival noted for any of the groups given AA immediately following the administration of T-2 toxin when compared to the control group given T-2 toxin and no therapy. Although the k-sample test indicated an overall significant difference in the survival curves for this study ($p \leq 0.025$), comparison of the individual treatment groups to the positive control group using Dunnett's multiple comparison test did not detect any significant differences in survival. However, the approximate median time to death determined from the survival curves for the 400, 800, and 1200 mg/kg groups were 25, 24, and 20 hr, respectively, while that for the positive control group was 32 hr.

The number of rats surviving and survival curves for rats pretreated with ascorbic acid at 400, 800, or 1200 mg/kg are shown in Table I.9 and Fig. I.23b, respectively. There was no overall improvement in survival between the AA treated groups and the group given T-2 toxin and no therapy. The approximate median time to death for each AA treatment group was 17 hr compared to 21.5 hr for the positive control group.

The groups in the AA studies given only the toxin vehicle + AA at 1200 mg/kg were not included in Fig. I.23 since all the animals survived the 72 hr observation period.

The number of rats surviving and survival curves for the control and treatment groups in the DEX + PGE_1 study are shown in Table I.10 and Fig. I.24, respectively. There was an overall significant difference detected in the survival curves using the k-sample test ($p \leq 0.025$). There was a significant improvement in survival between the positive control group (Group 1) and those groups given DEX alone or in combination with PGE_1 (Groups 2, 4, 5, and 6). No improvement was noted for the group given PGE_1 alone (Group 3). When the group given T-2 toxin and DEX alone (Group 2) was used as the control group and compared to the groups given the drug combination (Groups 1, 5, and 6) no significant differences were detected. Additionally, there was no indication of synergism when the combination was used. Of interest was the decrease in the median time to death of approximately 10 hr between the groups given DEX + PGE_1 at 25 and 50 µg/kg compared to the group given DEX alone; possibly indicating a detrimental effect on survival.

Discussion

Fricke and Jorge[18] reported that AA was efficacious for the treatment of acute T-2 toxicosis in mice. Mice treated with AA ip at 400, 800, or 1200 mg/kg followed immediately by T-2 toxin subcutaneously (sc) at 3.1 mg/kg (an approximate LD_{99} dose) had a significant improvement in survival compared to mice given T-2 toxin alone. The efficacy of AA for acute T-2 toxicosis in mice was hypothesized to be due to its antioxidant action.[a] Ascorbic acid may prevent the formation of oxygen-free radicals and subsequent membrane lipid peroxidation. The role that free radicals and lipid peroxidation play in the pathogenesis of acute T-2 toxicosis is unclear. It is evident from *in vitro* work that T-2 toxin at high concentrations can interact with cell membranes with subsequent alteration of membrane structure and function.[13,14] Segal and coworkers[29] studied the hemolytic activity of T-2 toxin using rat erythrocytes incubated with various high concentrations of T-2 toxin. The degree of hemolysis was correlated with toxin concentration. The hemolysis was noted to be similar to that caused by hydrogen peroxide and polyoxyethylene-derived surfactants which is believed to proceed via a free radical mechanism.[30,31] Darkness and free radical scavengers such as mannitol, histidine, glutathione and vitamin E attenuated the T-2 induced hemolysis. However, an attempt to verify the occurrence of lipid peroxidation by measuring malonaldehyde production yielded equivocal results. Tsuchida and coworkers[32] reported increases in thiobarbituric acid reactive substances in the liver, suggesting the occurrence of lipid peroxidation. In contrast, other tissues known to be more severely affected by T-2 toxin such as the gastrointestinal tract, spleen, and thymus did not contain significantly elevated concentrations of these products.

Additional evidence for a role for free radicals in the pathophysiology of acute T-2 toxicosis was suggested by the efficacy of 2 glutathione prodrugs,

L-2-oxo- (OTC) and L-2-methyl- (MTCA) derivatives of thiazolidine-4-carboxylate, given to mice prior to T-2 toxin administration.[19] Also, Fricke and Poppenga[a] reported that pretreatment of mice for 3 days with vitamin E ip at 120 mg/kg protected against T-2 toxin-induced lethality although the administration of vitamin E immediately or 4 hr after toxin administration was not effective in improving survival. The antioxidant food additive BHT was also effective if given as a pretreatment. However, other antioxidants such as ascorbyl palmitate, a more lipid soluble analog of ascorbic acid; n-propyl gallate; ethoxyquin; and Trolox®, a water soluble vitamin E analog, tested using mice[a] and the specific hydroxyl radical scavenger, dimethyl sulfoxide, evaluated in the rat,[17] were not of significant benefit in alleviating toxin-associated lethality.

The results of the present study confirmed earlier findings that showed AA to have no beneficial effect in the treatment of acute T-2 toxicosis in the rat.[17] Thus, there appears to be a species difference with regard to its efficacy. One possible factor is that, in rats, high doses of ascorbate cause a diminished plasma glucocorticoid response to stress.[33] This latter observation may account for the decrease in median survival times noted for the AA treated groups when compared to positive controls.

While still somewhat controversial, there appears to be ample evidence that glucocorticosteroids are of benefit for the treatment of other circulatory shock states.[34] The water-soluble salts of DEX or methylprednisolone are also efficacious in the treatment of acute T-2 toxicosis in experimental mice, rats, and swine. Dexamethasone given to rats iv at 1.6 mg/kg either 0.5, 1, or 3 hr after the administration of T-2 toxin iv at 0.75 mg/kg (an approximate LD_{66} dose) significantly improved animal survival.[16] Dexamethasone given to mice sc at 13 mg/kg either 1 hr prior to, at the same time as, or 1 or 2 hr after T-2 toxin given sc at 5 mg/kg (several times the LD_{50} dose) decreased lethality in all treatment groups.[15] Rats given T-2 toxin iv at 1 mg/kg (an approximate 1.5X LD_{50} dose) followed 15 min later by methylprednisolone sodium succinate iv at 30 mg/kg had significantly improved survival rates compared to control rats given T-2 toxin and no therapy.[17]

The efficacy of glucocorticosteroids appears to be due to the stabilization of cell membranes, particularly lysosomal membranes, with a resultant decline in the production of vasoactive substances such as myocardial depressant factor from ischemic organs, especially the pancreas.[34] There is no direct evidence for the role of lysosomal disruption and the production of MDF in the pathophysiology of acute T-2 toxicosis. However, pancreatic blood flow was severely compromised in swine given T-2 toxin iv at 2.4 mg/kg[7] and pancreatic lesions occurred in T-2 toxin dosed swine.[35] Glucocorticosteroids are also known to inhibit the production of prostaglandins via an inhibition of phospholipase A_2.[36] Thus, the production of detrimental prostaglandins such as thromboxane A_2, which increased in pigs dosed with T-2 toxin,[5] may be inhibited by glucocorticoids. Conversely, production of potentially useful prostaglandins, such as PGI and PGE_1, may also be reduced.

The efficacy of PGE_1 for the treatment of circulatory shock states has been demonstrated.[23,24] Salutary effects have been hypothesized to include vasodilation, improved cardiac function, and membrane stabilization with a resultant decrease in the release of lysosomal enzymes.[24]

The failure of the combination of DEX and PGE_1 to be more efficacious than DEX given alone may relate to several factors. The onset of circulatory shock induced by T-2 toxin appears to be slower than that following

administration of endotoxin. Thus, the timing of drug administration may be critical. Constant infusion of PGE_1 is required due to its rapid clearance from the circulation by the lung.[37,38] Therefore, physiological effects would be expected to subside rapidly following cessation of administration. We infused PGE_1 for 2 hr beginning immediately after T-2 toxin administration. This early administration of PGE_1 may not have allowed for maximal effect at the time of greatest need. Also, dexamethasone may have obviated the need for the PGE_1. Alternatively, the present study may indicate that the mechanisms involved in shock from T-2 toxin and those associated with endotoxic shock differ.

There was evidence for a detrimental effect on survival when the higher doses of PGE_1 (25 and 50 µg/kg) were used in combination with DEX compared to therapy with DEX alone. The reason(s) for this are not clear since the administration of PGE_1 alone at 50 µg/kg, while affording no benefit, was not detrimental compared to the positive control group given T-2 toxin and no therapy.

In summary, AA given 12 hr prior to and/or immediately after the administration of T-2 toxin did not prolong or improve survival in rats. On the contrary, AA may cause a decrease in survival. PGE_1 infused alone did not improve the survival of rats dosed with T-2 toxin and the addition of PGE_1 to therapy with DEX did not improve survival in rats over that obtained with DEX alone. In future work, the infusion of PGE_1 should be delayed following T-2 toxin administration until a later stage in the toxicosis. Since, to date, the water-soluble glucocorticosteroids appear to be the most efficacious agents for the treatment of animals with acute T-2 toxicosis, other therapies should be assessed in combination with the glucocorticoids in an effort to identify those which provide additional benefit.

Acknowledgements

The authors are grateful to Barbara Kindler, Mary Busse, and Tina Kerferlis for their excellent technical assistance. Our thanks also to Donna Lundeen for preparing the graphs and Sally Campbell for typing the tables. Special thanks to Dr. Ricardo Ochoa of The Upjohn Co. for graciously supplying the PGE_1.

These studies were supported in part by the U.S. Army Medical Research and Development Command, Contract No. DAMD 17-82-C-2179 and 17-85-C-5224. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

Footnotes

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[b] R. Ochoa, Upjohn Co., written communication.

[c] Harlan Sprague Dawley, Inc., Indianapolis, IN.

[d] University of Illinois, College of Veterinary Medicine. Toxicology Analytical Laboratory. Purity > 95% as assessed by gas chromatograpy with a flame ionization detector.

[e]Scorbic Acid Injection, The Butler Co., Columbus OH.

[f]Metofane, Pitman-Moore, Inc., Washington Crossing, NJ.

[g]Angiocath, Deseret Medical, Inc., Sandy, UT.

[h]One IU heparin per ml of normal saline.

[i]Azium-SP, 4 mg/ml dexamethasone sodium phosphate, Schering Corp., Kenilworth, NJ.

[j]Alprostadil, courtesy of The Upjohn Co., Kalamazoo, MI.

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Table I.8 Ascorbic acid treatment study: treatment groupings, dosing protocols, and survival data

Treatment Group	Dosing Protocol	No. of Survivors to 72 hr / Total No. of Animals in Group
1. T-2 toxin and saline	1 mg/kg T-2 toxin* iv** followed immediately by 1 ml saline per kg ip	1/7
2. Toxin Vehicle Control + Ascorbic Acid 1200 mg/kg	1 ml 50% ETOH:50% saline per kg iv** followed immediately by 1200 mg/kg ascorbic acid ip	10/10
3. T-2 Toxin + Ascorbic Acid 400 mg/kg	1 mg/kg T-2 toxin* iv** followed immediately by 400 mg/kg ascorbic acid ip	0/14
4. T-2 Toxin + Ascorbic Acid 800 mg/kg	1 mg/kg T-2 toxin* iv** followed immediately by 800 mg/kg ascorbic acid ip	2/14
5. T-2 Toxin + Ascorbic Acid 1200 mg/kg	1 mg/kg T-2 toxin* iv** followed immediately by 1200 mg/kg ascorbic acid ip	1/14

*T-2 toxin was dissolved in 50% ethanol:50% saline to a final concentration of 1 mg toxin per ml of vehicle.

**All iv injections were via a tail vein.

Table I.9 Ascorbic acid pretreatment study: treatment groupings, dosing protocols, and survival data.

Treatment Group	Dosing Protocol	No. of Survivors to 72 hr / Total No. of Animals in Group
1. T-2 Toxin and Saline	1 ml saline per kg ip 12 hr prior to 1 mg/kg T-2 toxin* iv** followed immediately by a second injection of saline ip	1/5
2. Toxin Vehicle Control + Ascorbic Acid 1200 mg/kg	1200 mg/kg ascorbic acid ip 12 hr prior to 1 ml 50% ethanol:50% saline per kg iv** followed immediately by a second injection of 1200 mg/kg ascorbic acid ip	5/5
3. T-2 Toxin + Ascorbic Acid 400 mg/kg	400 mg/kg ascorbic acid ip 12 hr prior to 1 mg/kg T-2 toxin* iv** followed immediately by a second injection of 400 mg/kg ascorbic acid ip	1/10
4. T-2 Toxin + Ascorbic Acid 800 mg/kg	800 mg/kg ascorbic acid ip 12 hr prior to 1 mg/kg T-2 toxin* iv** followed immediately by a second injection of 800 mg/kg ascorbic acid ip	1/10
5. T-2 Toxin + Ascorbic Acid 1200 mg/kg	1200 mg/kg ascorbic acid ip 12 hr prior to 1 mg/kg T-2 toxin* iv** followed immediately by a second injection of 1200 mg/kg ascorbic acid ip	1/10

*T-2 toxin was dissolved in 50% ethanol:50% saline to a final concentration of 1 mg toxin per ml of vehicle.

**All iv injections were via a tail vein.

Table I.10 Dexamethasone sodium phosphate + PGE_1 study: treatment groupings, dosing protocols, and survival data.

Treatment Group	Dosing Protocol	No. of Survivors to 72 hr / Total No. of Animals in Group
1. T-2 Toxin + Saline	1 mg/kg T-2 toxin* iv** followed by a sham saline injection iv,** a 2 hr saline infusion** at a rate and volume similar to that for PGE_1 treatment groups, and a second sham saline bolus injection iv** 4 hr after the first	1/9
2. T-2 Toxin + DEX	1 mg/kg T-2 toxin* iv** followed by dexamethasone iv** at 6 mg/kg, a 2 hr saline infusion at a rate and volume similar to that for PGE_1 treatment groups, and a second injection of dexamethasone iv** at 6 mg/kg 4 hr after the first	4/9
3. T-2 Toxin + PGE_1 (50 µg/kg)	1 mg/kg T-2 toxin* iv** followed by a sham saline bolus injection iv,** a 2 hr infusion** of PGE_1 for a total dose of 50 µg/kg, and a second sham saline bolus injection iv** 4 hr after the first	1/8
4. T-2 Toxin + DEX + PGE_1 (50 µg/kg)	1 mg/kg T-2 toxin* iv** followed by dexamethasone iv** at 6 mg/kg, a 2 hr infusion** of PGE_1 for a total dose of 50 µg/kg, and a second injection of dexamethasone iv** at 6 mg/kg 4 hr after the first	1/11
5. T-2 Toxin + DEX + PGE_1 (25 µg/kg)	As for group 4 except that a total dose of 25 µg/kg PGE_1 was infused** over a 2 hr period	4/12
6. T-2 Toxin + DEX + PGE_1 (5 µg/kg)	As for group 4 except that a total dose of 5 µg/kg PGE_1 was infused** over a 2 hr period	5/12

*T-2 toxin was dissolved in 50% ethanol:50% saline to a final concentration of 1 mg toxin per ml of vehicle.

**All iv injections and infusions were via an indwelling tail vein catheter.

Figure I 23a,b The effect of AA administered ip at 400, 800, or 1200 mg/kg immediately after or 12 hr prior to and immediately after iv administration of T-2 toxin at 1 mg/kg on the proportion of rats surviving over time. The symbols along the X-axis indicate the approximate median survival time for the corresponding treatment group.

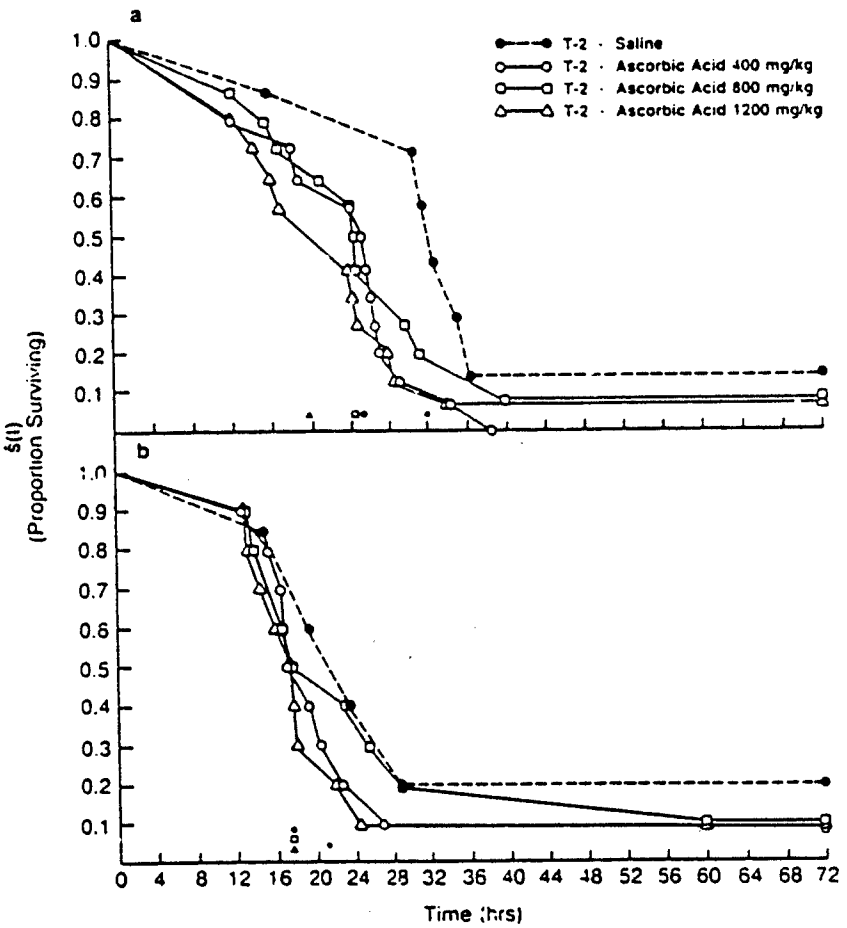
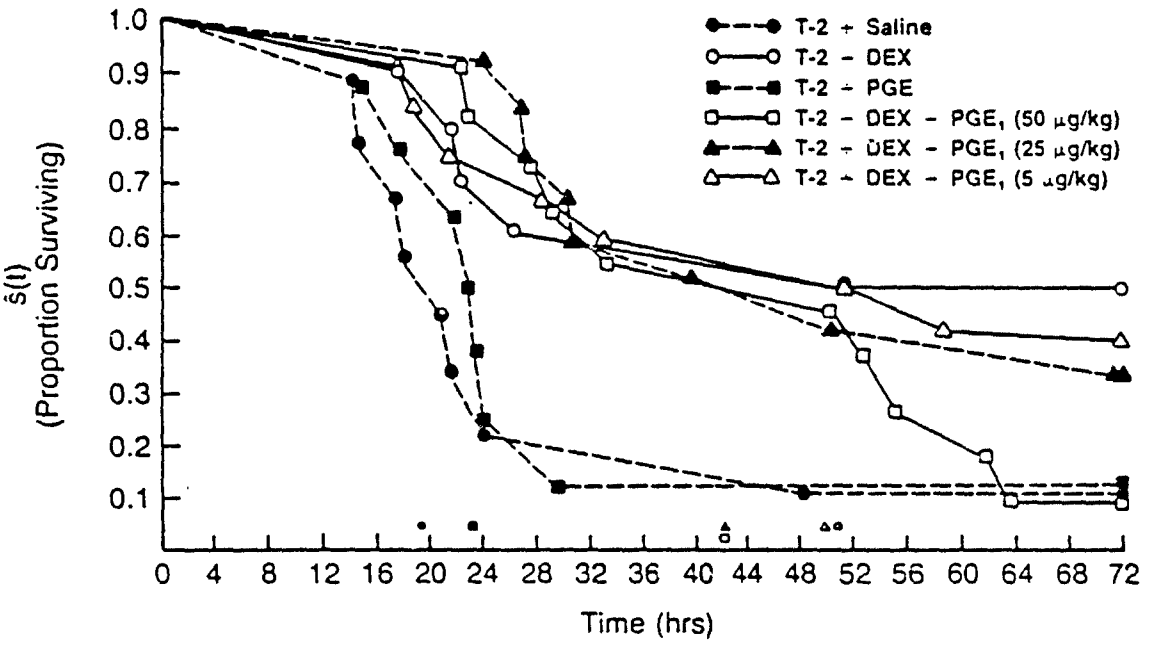


Figure I.24 The effect of DEX administered iv in combination with an infusion of PGE1 immediately after the iv administration of T-2 toxin at 1 mg/kg on the proportion of rats surviving over time. The symbols along the X-axis indicate the median survival time for the corresponding treatment group.



E. Distribution of Blood Flow to the Gastrointestinal Tract of Swine During T-2 Toxin-Induced Shock

by
Val Richard Beasley, Gregg R. Lundeen, Robert H. Poppenga, William B. Buck, and William J. Tranquilli

Abstract

Three groups of swine (6 per group) were used to study blood flow alterations associated with T-2 toxin-induced shock. Low and high dose groups were given T-2 toxin at 0.6 or 2.4 mg/kg, respectively; the third group served as a vehicle (70% ethanol) control. Radiolabeled microspheres were administered into the left atrium to assess organ blood flow predosing and at 90-minute intervals until 6 hours postdosing.

Gastric blood flow was reduced postdosing in both T-2 toxin-dosed groups, but the reduction was more severe at the high dose. At 6 hours, the gastric blood flow of the high dose group had declined to 17% of this group's predose value. The maximal reduction in gastric blood flow of the low dose group, to 30% of the group's predose value, was observed at 3 hours postdosing; by 6 hours, it had increased to 49%.

Small intestinal blood flow of the control group gradually declined over the 6 hours to 64% of the predose value. The high dose group displayed a maximal increase in small intestinal blood flow at 3 hours to 174% of the predose value. This was followed by a reduction at 6 hours to 62% of the predose value, at which time the swine were experiencing a severe decline in cardiac output to 37% of the predose value. Blood flow to the small intestine of the low dose group was increased approximately 60% at 1.5 hours and then gradually decreased to the control value over the remainder of the observation period.

The large intestinal blood flow of the high dose group increased at 3 hours to 177% of the predose value and declined, but only very late in the experiment, as indicated by a mean of 100% of the predose value at 4.5 hours followed by 66% at 6 hours. The large intestinal blood flow of the low dose group, however, was increased to 151, 196, 200, and 142% of the predose value for this group at 1.5, 3, 4.5, and 6 hours, respectively.

The reduction in gastric blood flow is likely to be related to the frequent development in swine given high doses of T-2 toxin and of a grossly bright red gastric fundus with histologic evidence of vascular congestion in the submucosa and mucosal degeneration and necrosis. The increased blood flow in the intestine may account for the lesser susceptibility of these areas to the acute effects of the toxin.

Introduction

The trichothecene mycotoxin, T-2 toxin, is believed to be produced primarily by species of Fusarium. It is capable of causing significant poisoning in livestock (Greenway and Puls, 1976) and apparently humans (Joffe, 1983) exposed to naturally contaminated grains. T-2 toxin, other trichothecenes, and another Fusarium toxin, zearalenone, have also been implicated as components of a purported chemical warfare agent, often referred to as "yellow rain" (Rosen and Rosen, 1982; Mirocha et al., 1983). The toxin is capable of causing damage or dysfunction in the digestive tract (Weaver et

al., 1979a), immune (Rafai and Tuboly, 1982), and hematopoietic (Hayes et al., 1980) systems; heart and pancreas (Pang et al., 1986); nervous system (Feuerstein et al., 1985); integumentary system (Marasas et al., 1969); and reproductive tracts of both males (DiNicola et al., 1978) and females (Weaver et al., 1978b).

After oral exposure to trichothecenes in naturally contaminated grains (Greenway and Puls, 1976) via feeds containing crude or semipurified toxins (Mortimer et al., 1971) or following administration of semipurified or purified toxins via oral (Lutsky et al., 1978) or intravenous (Yap et al., 1979) routes, serious clinical signs and lesions referable to the gastrointestinal tract are among the most prominent effects. In T-2 toxin-exposed swine, clinical signs may include perioral and oral inflammation (after ingestion only) (Rafai and Tuboly, 1982), vomition, redness of the stomach, and an increasing vascular congestion as the small intestine is examined going from the duodenum to the terminal ileum (Weaver et al., 1978a).

T-2 toxin is apparently absorbed comparatively slowly from the stomach (Beasley et al., 1986), but it is rapidly absorbed upon entering the small intestine. Its metabolism involves ester hydrolysis (Knupp et al., 1986) and hydroxylation (Yoshizawa et al., 1982). T-2 dosed swine have higher relative ratios of unconjugated vs. conjugated metabolites in the large intestine as compared to ratios in the small intestine, suggesting bacterial deconjugation in the large colon (Corley et al., 1986). Notable detoxification, however, is most likely attributable to gluconate conjugation (Corley et al., 1986) as well as epoxide reduction by digestive tract microbes (Yoshizawa et al., 1985; Swanson et al., 1987).

At comparatively high doses (0.6 mg/kg and greater), T-2 causes circulatory shock; hemodynamic effects and the systemic distribution of blood flow of dosed swine have been reported (Lorenzana et al., 1985; Lundeen et al., 1986). Local effects of this toxin on blood flow to these organs are of interest in view of the pronounced clinical signs and lesions referable to the digestive tract and emerging information on the toxins disposition in the body. The present report, therefore, further delineates blood flow in swine dosed with T-2 toxin at both sublethal and potentially lethal doses. Swine were chosen as our experimental model because of physiologic and anatomic similarities with humans (Dodds and Hsu, 1982).

Materials and Methods

Eighteen healthy female pigs weighing 43.5 to 97.5 kg (54.7 $\pm$ 4.4 kg; mean $\pm$ SEM) were used in this study. Each animal was surgically instrumented several weeks prior to beginning the study. Most of the experimental procedures used in this study have been described in detail previously (Lundeen et al., 1986).

The T-2 toxin used was prepared in our laboratory from extracts of *Fusarium sporotrichioides* grown on rice culture and was of 95% purity as determined by gas chromatography with flame ionization detection of the corresponding trimethylsilylether derivative.[1]

Surgical Preparation of Animals

Animals were anesthetized with a mixture of halothane and oxygen, and saline-filled Tygon catheters were implanted in the left atrium, pulmonary

artery, ascending aorta via the internal thoracic artery, and the descending aorta via the femoral artery; tunneled subcutaneously; and then heparinized.

After a period of 4 to 8 weeks, the animals were restrained using a sling, and the catheter ends were removed from their subcutaneous locations after local administration of lidocaine.

Procedures

Cardiac output was determined in triplicate using the dye dilution technique and by the microsphere technique as previously described (Lundeen et al., 1986). Standard criteria for organ blood flow and cardiac output determinations by the microsphere method (Buckberg et al., 1971; Archie et al., 1973) were satisfied. Microspheres[2] (15 μm; radiolabelled with ^{141}Ce, ^{113}Su, ^{103}Ru, $^{95}N6$, or ^{46}Sc) were injected into the left atrium for organ blood flow determinations. The microspheres were randomly assigned among the time points for each animal. At the end of the experiment (shortly after the 6-hour time point), the pigs were anesthetized with sodium thiamylal and killed by exsanguination. The stomach, small intestine, and large intestine were removed and cleared of mesenteries and visceral peritonium. The organs were minced, placed in preweighted vials, weighed again, and counted in a gamma well scintillation counter[3] in the same manner as reference arterial blood samples.

Protocol

Treatments were randomly assigned to the animals so that by the end of the study, 6 animals were present in each of 3 treatment groups: (1) ethanol control, (2) low dose T-2 toxin (0.6 mg/kg) and, (3) high dose T-2 toxin (2.4 mg/kg). After predosing (0 hour) dye-dilution cardiac output measurement and an infusion of microspheres for organ blood flow determinations, 7 ml of 70% ethanol (vehicle control), T-2 toxin at 0.6 mg/kg in 7 ml of 70% ethanol (low dose), or T-2 toxin at 2.4 mg/kg in 7 ml of 70% ethanol was infused into the pulmonary artery over a 2-minute period. Dye-dilution cardiac output determinations and an infusion of microspheres were then repeated every 1.5 hours up to 6.0 hours after toxin administration.

Measurements and Calculations

Organ blood flow values were derived as described previously (Lundeen et al., 1986).

Statistical Analysis

A Wilk's Lambda criterion transformed to an F-statistic was used to evaluate the overall effect of time and the interaction between time and treatment.[4] Within subjects, effect of time was also decomposed into polynomial trends (linear, quadratic, cubic, and quartic). In instances in which significant time treatment interactions were observed, one-way analyses of variance were used to compare treatment groups at individual time points. For all analyses, a level of significance of $\alpha = 0.05$ was chosen.

Results

Values for blood flow to the stomach, small intestine, and large intestine are shown in Figures I.25 to I.27. Cardiac output values are illustrated in

Figure I.28. For each organ, predosing blood flow values among the 3 groups were very similar.

Cardiac Output

Cardiac output determinations using the microsphere method were within 4 to 6% of the dye-dilution values simultaneously obtained. In the control group, a slight decrease in CO was observed at 1.5 and 3 hours postdosing to a low value of 90% of the predose cardiac output. Thereafter, a steady increase to 104% of the predose value at 6 hours was observed. In the group given the low dose of T-2 toxin, an initial modest increase was followed by a decline to 72% of the predose value at 3 hours and a partial return toward the predose value thereafter. The high dose group displayed a precipitous drop in cardiac output to values of 90, 59, 41, and 37% of the predose values at 1.5, 3, 4.5, and 6 hours after dosing, respectively.

Stomach

There was a significant ($p = 0.0001$) effect of time on gastric blood flow. More importantly, there was a significant ($p = 0.0004$) interaction between time and treatment. The analysis via decomposed polynomials revealed an overall quadratic trend with regard to time ($p = 0.0001$) which also differed among treatment groups ($p = 0.0002$). When one-way analyses of variance at each time point were used to compare treatment groups, no differences were detected at the predosing time point; a significant ($p = 0.048$) difference was apparent between groups at 1.5 hours, and highly significant differences ($p < 0.0001$) were apparent at all subsequent time points.

The maximal change in mean gastric blood flow of the control groups was the reduction at 1.5 hours to 84% of the predose value was followed by a slight increase toward the initial value. Postdosing reductions in gastric blood flow of the two T-2-dosed groups were almost identical until 3 hours; at that time, the low and high dose group values were 30 and 33% of their predosing values, respectively. Thereafter, the value of the low dose group returned toward normal reading 50% of the control value at 6 hours. The high dose group continued its decline so that at 4.5 and 6 hours the gastric blood flow was only 17%, respectively, of the group's initial value.

Small Intestine

For small intestinal blood flow across all treatment groups, there was an overall significant effect of time ($p = 0.0013$). There was also a significant ($p = 0.0148$) interaction between time and treatment. The analysis via decomposed polynomials revealed an overall quadratic trend with regard to time ($p = 0.0001$). The different group displayed significantly different ($p = 0.0420$) quartic trends with regard to time. The analyses of variance comparing the treatment groups at individual time points revealed a significant ($p = 0.0060$) difference in small intestinal blood flow only at 3 hours although values of $p = 0.0833$ and $p = 0.0617$ were obtained for 1.5 and 4.5 hours, respectively.

Small intestinal blood flow of the control group gradually declined so that at 6 hours the value was 64% of the initial value. Both T-2-treated groups displayed transient elevations in small intestinal blood flow. The maximal increase for the low dose group to 159% of the predose value was reached at 1.5 hours; thereafter, blood flow declined to very near the predose value at 6 hours. In the high dose group, an initial transient decrease in blood flow

to the small intestine at 1.5 hours was followed by a maximal value at 3 hours of 175% of the predose value. This increase was followed by a drop in blood flow so that, at 6 hours, blood flow to the small intestine was equivalent to that of the control group.

Large Intestine

As with the gastric and small intestinal blood flow effects, there was a significant ($p = 0.0007$) overall effect of time and a significant ($p = 0.0132$) interaction between time and treatment. Decomposition via polynomial trends revealed a significant overall quadratic trend with regard to time ($p = 0.001$) which differed among treatment groups ($p = 0.0181$). The application of one-way analyses of variance at each time point revealed no significant differences predosing ($p = 0.4645$) or 1.5 hours ($p = 0.1051$), but significant differences were detected at 3 ($p = 0.0410$), 4.5 ($p = 0.0205$), and 6 hours ($p = 0.0141$) postdosing.

The large intestinal blood flow of the control group remained near normal with the maximal change, at 6 hours, consisting of a reduction to 88% of the initial value. By contrast, the low dose group exhibited steadily increased large intestinal blood flow until 4.5 hours after T-2 toxin administration when it reached 201% of the predosing value. At 6 hours, the flow had declined to 142% of the predose value. In the high dose group, an inital modest reduction was followed by an increase at 3 hours, to 130% of the initial value and a final reduction to 56% at 6 hours.

Discussion

Previous reports on values from pigs given acutely toxic doses of T-2 toxin have indicated that the toxic shock syndrome produced is accompanied by vomition, flattulence, abdominal rigidity on exhalation, a drop in cardiac output metabolic acidosis, and increased circulating concentrations of epinephrine, norepinephrine, thromboxane B-2, and 6-keto-$PGF_{1\alpha}$ (Lorenzana et al., 1985; Lundeen et al., 1986). In the same swine as described in this report, the most pronounced reductions in blood flow were those to the spleen and pancreas (Lundeen et al., 1986). In contrast, the reduction in blood flow to the kidneys paralleled the drop in cardiac output, while the more modest declines in blood flow to the heart and brain appeared to reflect compensatory mechanisms. Despite the drop in cardiac output, postdosing elevations in blood flow were observed for the adrenal glands, liver (arterial), and overall gastrointestinal tract.

The present study revealed that in spite of the overall increase in total gastrointestinal blood flow, the gastric blood flow had declined at a rate faster than the drop in cardiac output, particularly at the first (1.5 hour) time point but continuing thereafter. Gastric perfusion of the high dose animals was severely compromised. The reduction in gastric blood flow may contribute to the poor absorption of T-2 toxin from this organ (Beasley, 1983).

It is important to note that, although swine often vomit after being given T-2 toxin, all cardiac output determinations and microsphere administrations were timed so that they did not coincide with episodes of vomiting. In view of the rapid effect of T-2 toxin on gastric profusion, however, it is possible that reduction in blood flow was a cause of gastritis that may contribute to T-2-associated vomiting. Vomiting, associated with the administration of another trichothecene mycotoxin, fusarenone-X has

previously been attributed to stimulation of two chemoreceptor trigger zones, but this interpretation was based soley on the ability to prevent vomiting by means of administration of chlorpromazine, an agent known to depress the emetic center. In the absence of further data, it cannot presently be stated whether affects on the chemoreceptor trigger zone or reductions in gastric blood flow are individually or jointly responsible for the vomiting observed in trichothecene-exposed animals.

It is apparent that increased hepatic artery blood flow combines with an increase in portal flow (due to elevations in small and large intestinal perfusion) to provide a substantial increase in circulation through the liver. This is likely to contribute to preferentially provide the liver with oxygen, nutrients, the parent compound, and reabsorbed metabolites from the intestine to permit the observed, rapid initial, and subsequent steps in metabolism (Beasley et al., 1986; Corley et al., 1986). In addition, since it appears that bacterial deconjugation of glucuronide metabolites occurs in the large intestine (Corley et al., 1986), the local increase in perfusion may contribute to reabsorption of free (and therefore toxic) initial metabolites of T-2 toxin, as well as unconjugated but much less toxic de-epoxy metabolites.

Other workers have speculated that the gastrointestinal mucosal lesions observed in trichothecene-exposed animals are due to a primary "radiomimetic" effect of the toxin on the rapidly dividing epithelial cells (DeNicola et al., 1978; Coppock et al., 1985) or to "well-recognized irritant toxicity" (Hayes et al., 1980). In one of these reports, however, an increased number of microthrombi in the cecum was associated with an increased degree of enterocyte damage (Coppock et al., 1985). Moreover, rats given repeated, acutely toxic doses of T-2 toxin displayed lesions in the left auricle and ventricle, kidneys, pancreas, and testis, all of which were associated with arterial pathology and a degree of arterial occlusion (Schoental et al., 1979; Wilson et al., 1982; Yarom et al., 1983). Finally, T-2 toxin has been studied using an isolated bovine ear preparation, and its effects (at high rates of perfusion) included vasoconstriction which was not affected by the presence of norepinephrine or histamine and not responsive to inhibition by either an antihistamine or a beta-adrenergic antagonist (Wilson and Gentry, 1985).

In agreement with the reports suggesting blood flow impairment, workers in our laboratory have correlated local reductions in blood flow with the appearance of splenic, pancreatic, and myocardial lesions in trichothecene-dosed swine (Pang et al., 1986; Lundeen et al., 1986). Similarly, in view of the severity of the ischemia in the stomach, the possibility of a major contribution of hypoperfusion to gastric mucosal damage cannot be ignored. Whether the disproportionate reduction in blood flow to the stomach was a result of primary or secondary vasoconstriction, vascular thrombi or primary (cytotoxic) endothelial damage was not determined. Reduced flow has been previously implicated as a cause of gastric lesions in shock and has been associated with reduced clearance of back-diffusing hydrogen ions (Kivilaakso et al., 1978). In addition, gastric lesions in rats given endotoxin were reduced by administration of naloxone or proglumine (Parmer, 1986), suggesting the possible involvement of endorphins or gastrin, respectively.

With the sublethal (0.6 mg/kg) dose of T-2 toxin, the initial increase in blood flow to the small and large intestine may account for the comparatively mild lesions usually observed in these organs in swine given this dose. In pigs given potentially lethal doses, however, the late declining trends in

both cardiac output and gastrointestinal blood flow are likely to continue until death, which may not occur until 12 hours or later. In all likelihood, this eventually results in significant hypoperfusion to the gut and contributes to the development of more notable intestinal lesions which may develop during lethal trichothecene poisoning.

In summary, it seems clear that local digestive system blood flow influences toxin disposition. In addition, a combination of cytotoxic and, especially, ischemic effects influence the development of the gastrointestinal lesions associated with T-2 toxin induced shock.

Footnotes

[1]T-2 toxin produced and purified by Steven P. Swanson, MS, and Harold Dean Rood, Jr., BS.

[2]New England Nuclear/Dupont, Boston, MA.

[3]Gamma Trac 2250, TM Analytic, Elk Grove, IL.

[4]SAS Institute, Inc., Cary, NC.

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Figure I.25 Cardiac output (mean ± SE) of swine given T-2 toxin at 0 (•), 0.6 (○), and 2.4 (△) mg/kg.

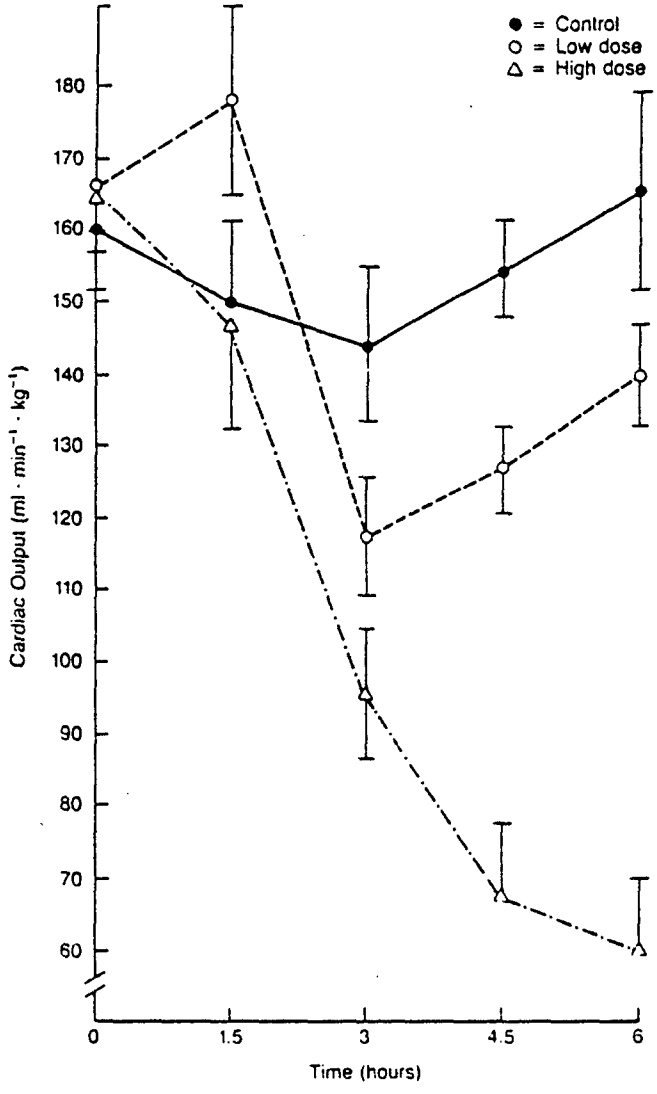


Figure I.26 Gastric blood flow (mean ± SE) of swine given T-2 toxin at 0 (•), 0.6 (○), and 2.4 (Δ) mg/kg.

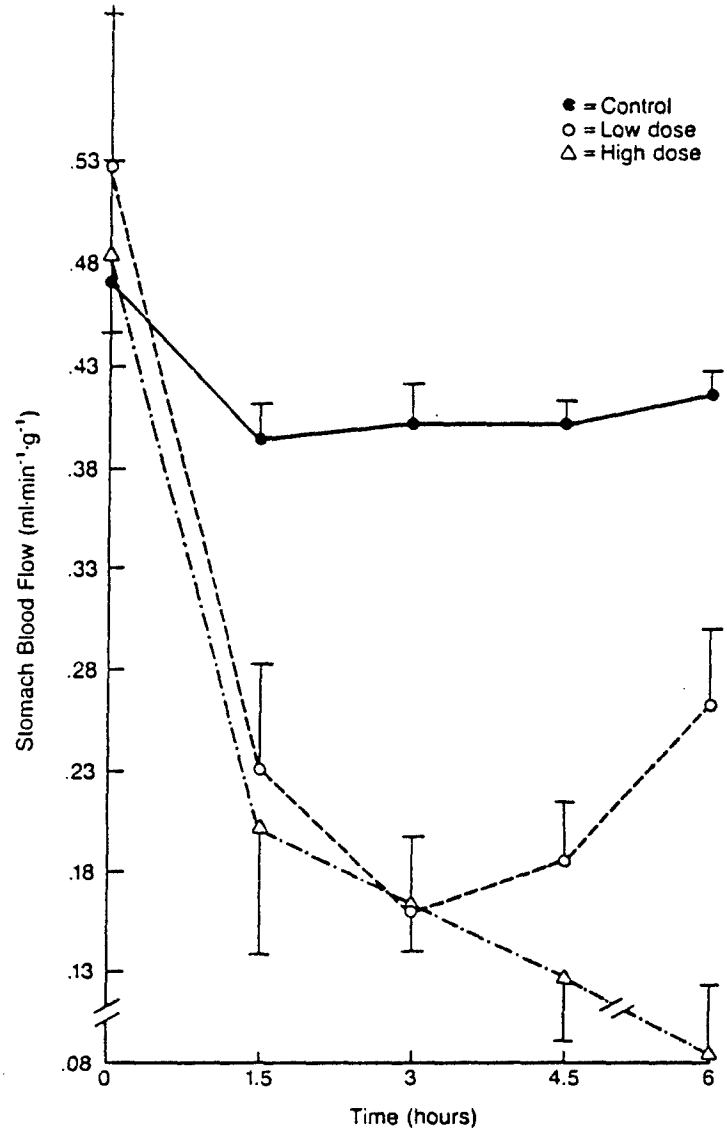


Figure I.27 Small intestinal blood flow (mean ± SE) of swine given T-2 toxin at 0 (•), 0.6 (○), and 2.4 (Δ) mg/kg.

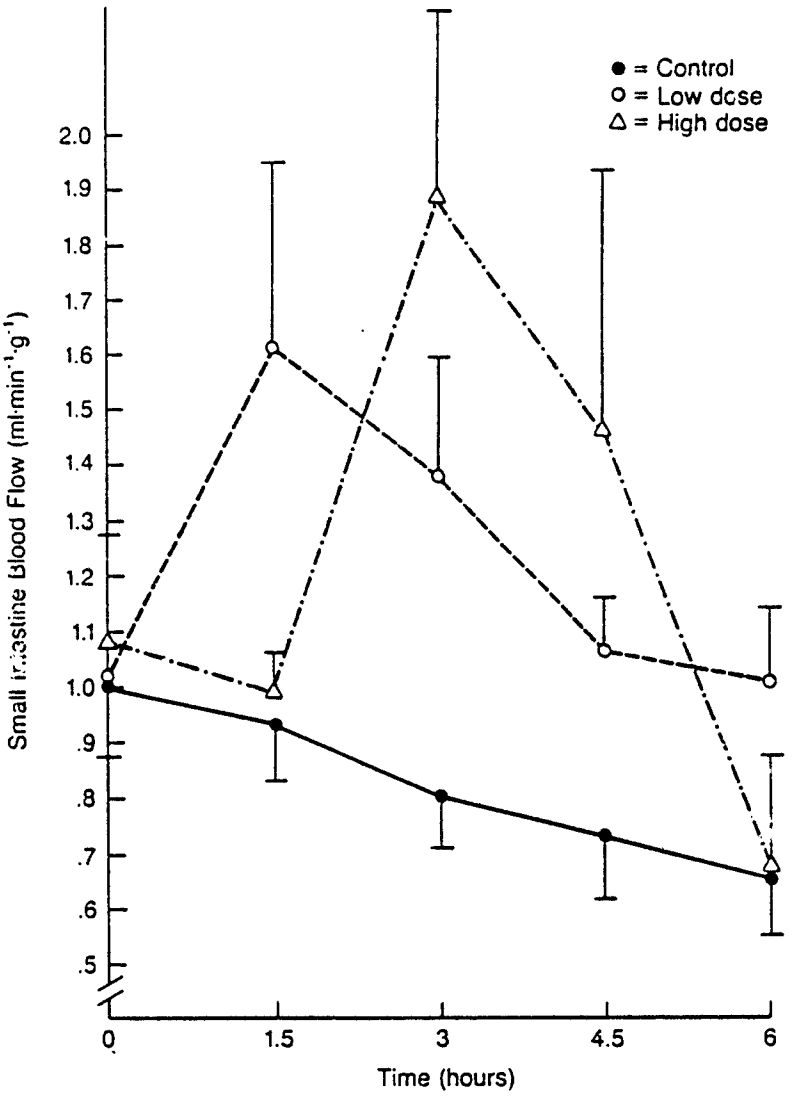
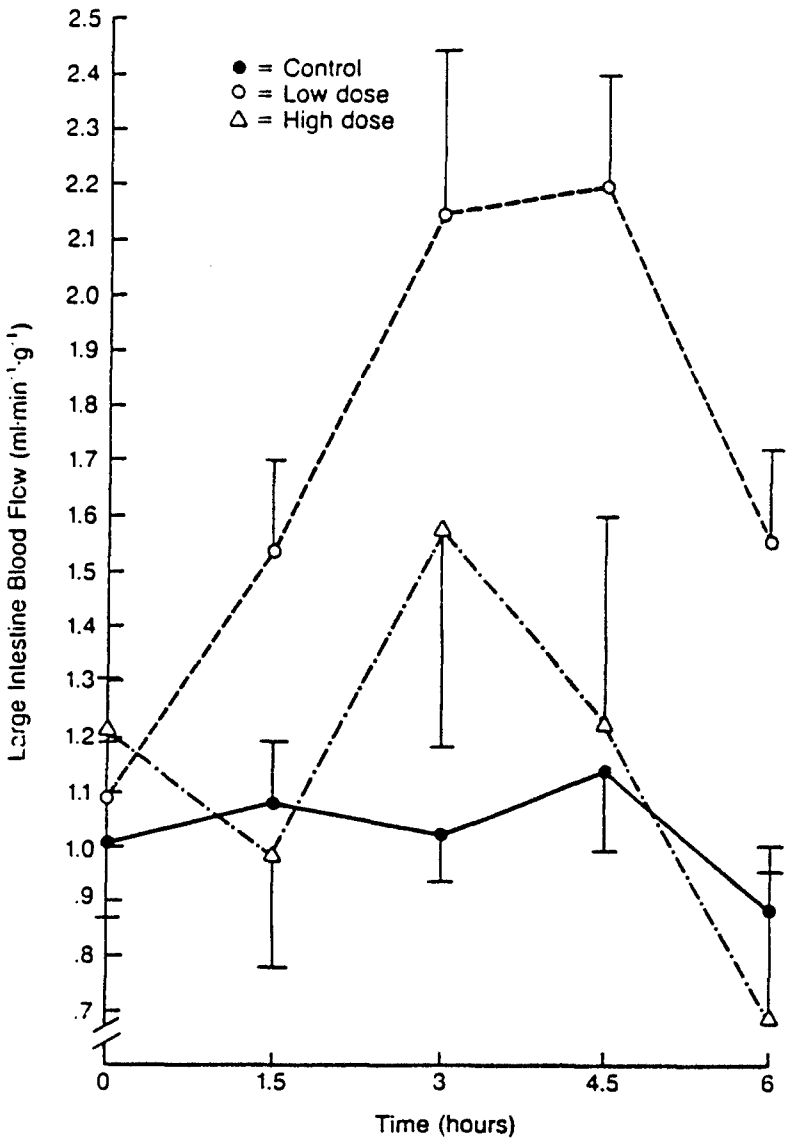


Figure I.28 Large intestinal blood flow (mean ± SE) of swine given T-2 toxin at 0 (•), 0.6 (○), and 2.4 (Δ) mg/kg.



F. Oral Superactivated Charcoal Studies in Swine

by
Kathy A. Coddington, William B. Buck, and Richard J. Lambert

Studies done in our laboratory indicated that orally administered superactivated charcoal prolonged the survival time of rats given lethal oral doses of T-2 toxin. Swine were chosen as an experimental model for further efficacy testing of superactivated charcoal due to the similarities in gastrointestinal function between humans and swine.

In order to confirm an oral dose of T-2 toxin which would result in death in small pigs (11 to 20 kg), 2 pigs were dosed at 3.6 and 8 mg/kg. The pig given 3.6 mg/kg of T-2 toxin survived but developed clinical signs of protracted vomiting, profuse diarrhea, and subnormal temperature. The pig given 8 mg/kg of T-2 toxin became symptomatic and died within 18 hours.

In veterinary and human medicine, cathartics are commonly administered along with activated charcoal to promote the excretion of toxic substances bound to the charcoal. Rat studies in our laboratory (Galey et al., 1986) had shown that when compared to superactivated charcoal alone, an osmotic cathartic was of no additional benefit in prolonging the survival time of rats given oral lethal doses of T-2 toxin. Sorbitol, when administered with superactivated charcoal, was shown to be detrimental to rodent survival. We decided to determine the efficacy of 3 cathartics and superactivated charcoal as a preventative therapy for swine that had received lethal doses of T-2 toxin.

Two oral studies were done to evaluate superactivated charcoal and selected cathartics. In study one, 12 crossbred female swine were dosed as in Table I.11. Pertinent clinical observations for these swine are reported in Table I.12. In phase two, 15 crossbred female swine were dosed as in Table I.13. Clinical signs, survival time, and gross pathologic lesions were evaluated (Tables I.14 and I.15).

Clinical signs and death occurred in each of the swine given 10 mg/kg T-2 toxin orally but no therapy. Swine receiving no therapy died over a range of 11 to 37 hours postdosing. All of the swine receiving superactivated charcoal alone or in combination with a cathartic survived to the end of the observation interval (5 days or 2 days). Treated swine were active, alert, and eating within 8 hours after dosing.

Superactivated charcoal or superactivated charcoal and cathartics (sorbitol, magnesium sulfate, or sodium sulfate) when given within 2 minutes of T-2 oral dosing (lethal dose of 10 mg/kg) were of benefit in alleviating clinical signs of T-2 toxicosis.

There were no apparent detrimental effects caused by the administration of a cathartic with superactivated charcoal, which was in contrast with results obtained in the rat model. Additionally, gross lesions associated with oral exposure to T-2 toxin were minimal or absent in swine treated with superactivated charcoal or superactivated charcoal and sorbitol.

Table I.11 Superactivated charcoal therapy for oral T-2 exposure in swine (Study 1 dosing schedule and survival)

Group	Number of Gilts	Time Interval following T-2 Dosing to Therapy	Survival (5 days)
1. T-2 and water	2	water < 1 minute (no therapy)	0
2. T-2 and superactivated charcoal	2	< 1 minute	2
3. T-2, superactivated charcoal, and sorbitol	2	< 1 minute	2
4. T-2 superactivated charcoal, and magnesium sulfate	2	< 1 minute	2
5. T-2, superactivated charcoal, and sodium sulfate	2	< 1 minute	2

T-2 toxin ($\geq$ 95% purity) was dosed orally at 10 mg/kg via stomach tube (50% w/v solution in ethanol).

Superactivated charcoal and the following cathartics were dosed at 1 gm/kg of body weight (20% w/v suspension in water).

1. Superactivated charcoal (Gulf Biosystems, Inc., Dallas, TX)
2. Magnesium sulfate
3. Sodium sulfate

Sorbitol was dosed at 700 mg/kg via stomach tube (commercially prepared suspension of superactivated charcoal and sorbitol, Gulf Biosystems, Inc., Dallas, TX).

Table I.12 Superactivated charcoal therapy for oral T-2 exposure in swine (Study 1 results).

Group	Therapy Administered*	Incidence of Emesis	Onset of Emesis[1]	Time Period During which Emesis Occurred[1]	Trend in Body Temperature Change	Change in Rectal Temperature °C
1	N	9	13	66	decrease	7.0
1	N	18	22	99	decrease	3.7
2	SAC	0	0	0	increase	0.8
2	SAC	4	56	348	decrease	1.5
3	SAC, S	1	60	0	increase	1.8
3	SAC, S	1	420	0	increase	0.9
4	SAC, MS	1	65	0	increase	0.8
4	SAC, MS	1	240	0	increase	0.7
5	SAC, SS	3	3	44	increase	1.3
5	SAC, SS	1	60	0	increase	1.6

[1]In minutes.

*Therapy Administered:

N = None (T-2 toxin positive control given water to approximate therapy volume)
SAC = Superactivated charcoal
S = Sorbitol (700 mg/kg)
MS = Magnesium sulfate (1 gm/kg)
SS = Sodium sulfate (1 gm/kg)

Table I.13 Superactivated charcoal therapy for oral T-2 exposure in swine (Study 2 dosing schedule and survival)

Group	Number of Gilts	Time Interval following T-2 Dosing to Therapy	Survival (48 hours)
1. T-2 and water	5	water only (no therapy)	0
2. T-2 and superactivated charcoal	5	2 minutes	5
3. T-2, superactivated charcoal, and sorbitol	5	2 minutes	5

Swine were dosed orally with T-2 toxin ($\geq$ 95% purity) at 10 mg/kg of body weight via stomach tube (50% w/v solution in ethanol).

Superactivated charcoal was dosed at 1 gm/kg body weight via stomach tube (20% w/v suspension in water).

Sorbitol was dosed at 700 mg/kg of body weight via stomach tube (commercially prepared suspension of superactivated charcoal and sorbitol, Gulf Biosystems, Inc.).

Table I.14 Superactivated charcoal therapy for oral T-2 exposure in swine (Study 2 results)

Group	Therapy Administered*	Animal Number	Incidence of Emesis	Onset of Emesis after T-2 Dosing[1]	Time Period during which Episodes of Emesis Occurred[1]
1	N	1	24	4	93
1	N	2	25	8	879
1	N	3	23	10	604
1	N	4	36	5	875
1	N	5	21	2	199
2	SAC	6	5	25	60
2	SAC	7	9	7	99
2	SAC	8	10	15	103
2	SAC	9	11	14	89
2	SAC	10	11	10	75
3	SAC, S	11	5	34	45
3	SAC, S	12	4	14	24
3	SAC, S	13	2	22	32
3	SAC, S	14	none	none	none
3	SAC, S	15	5	39	34

[1]Minutes.

*Therapy Administered:

N = None (T-2 toxin, 10 mg/kg orally; given water to approximate therapy volume)
SAC = Superactivated charcoal (1 gm/kg)
S = Sorbitol (700 mg/kg)

Table I.15 Gross necropsy results for Study 2

Organ/Finding	Group 1 T-2	Group 2 T-2 and Superactivated Charcoal	Group 3 T-2, Superactivated Charcoal, and Sorbitol
Brain			
Congestion of meninges, diffuse	5/5	1/5	0/5
Stomach			
Hemorrhage/necrosis, diffuse (glandular portion)	5/5	0/5	0/5
Erosion, linear, focal	0/5	1/5	1/5
Small and Large Intestine			
Congestion, diffuse	5/5	1/5	0/5
Fluid Contents	5/5	0/5	0/5
Skin, Snout, Ears			
Purple discoloration	5/5	0/5	0/5

G. Evaluation of a Superactive Charcoal Paste and Detergent and Water in Prevention of T-2 Toxin-Induced Local Cutaneous Effects in Topically Exposed Swine

by
Michael L. Biehl, Richard J. Lambert, Wanda M. Haschek,
William B. Buck, and David J. Schaeffer

Abstract

T-2 toxin (6 mg) dissolved in 90% DMSO was topically applied to nine 9-cm^2 sites on the dorsum of each of 9 young, crossbred, SPF, female pigs, 20.6 $\pm$ 1.9 kg in weight. A superactive charcoal paste (SAC) and/or a soap and water wash (SOAP) was applied to 8 of the T-2 exposed sites on each animal. These treatments were applied at various times postexposure ranging from 5 to 65 minutes. The site which received T-2 alone served as a positive control. DMSO was applied to a tenth site on each pig as a negative control. Animals were killed on 1, 3, or 6 days after treatment. Skin lesions were examined and graded grossly and histologically. No adverse systemic clinical signs were observed in any of the animals. Marked reddening and slight swelling of the T-2 toxin treated positive control sites was present throughout the study. Ulceration of this site was first noted on Day 3. All therapeutic regimens effectively reduced lesion severity resulting from T-2 toxin application. Significant differences in relative effectiveness were also seen between treatments. In each significant pair, the ordering of mean lesion severity was SAC/SOAP < SAC or SOAP and SOAP < SAC. As a single treatment, SOAP appears to be more effective than SAC in reducing lesion severity. These results failed to provide unequivocal evidence of an additive therapeutic effect when SAC and SOAP were used sequentially on the same site.

Introduction

T-2 toxin [4,15-diacetoxy-8-(3-methylbutyloxy)-12,13-epoxy-Δ^9-trichothecen-3-ol] and other mycotoxins of the trichothecene family are highly irritating to skin and mucous membranes of both man and animals (Forgacs and Carll, 1962; Forgacs, 1972; Wyatt, 1972; Smalley, 1973; Rodricks and Eppley, 1974; Pier et al., 1976; Weaver et al., 1977; Weaver et al., 1978; Weaver et al., 1981). Laboratory workers accidentally exposed to cultures of trichothecene-producing fungi exhibited mild cutaneous irritation, edema, and inflammation, which was followed by desquamation (Mortimer et al., 1971). This propensity for dermal irritation led to the use of laboratory animal skin bioassays as a means of semiquantitatively testing for the presence of trichothecene mycotoxins in feedstuffs (Gilgan et al., 1966; Ueno et al., 1970; Wei et al., 1972; Chung et al., 1974; Hayes and Schiefer, 1979).

More recently, international attention has been focused on the alleged use of trichothecenes as chemical warfare agents in Southeast Asia and Afghanistan (Holden, 1982; Rosen and Rosen, 1982; Mirocha et al., 1983). Since most of these reports have described aerial release as the method of delivery, skin contact would be an important route of exposure.

We have used pigs to study the pathopysiologic effects of T-2 toxin following topical exposure (Pang et al., 1987). Compared to the skin of other mammals frequently used for dermal studies (mice, rats, rabbits, and guinea pigs), swine skin more nearly resembles human skin anatomically and functionally (Weinstein, 1966; Marzulli et al., 1969; Bartek et al., 1972; Bisset and McBride, 1983; Hawkins and Reifenrath, 1984; Reifenrath et al., 1984).

Therapeutic methods are needed to minimize the severity of human cutaneous lesions caused by dermal exposure to tricothecene toxins. Application of T-2 toxin to the skin produces a number of effects which we have broadly grouped as "pathological" or histopathological. Superactivated charcoal is efficacious in treating rats exposed orally to T-2 toxin (Buck and Bratich, 1986; Galey et al., 1987). Our primary purpose was to evaluate the therapeutic effectiveness of a superactive charcoal paste and a soap and water wash, either alone or as a combined therapy, in altering the complexus of effects represented by these groups, singly and jointly. A secondary objective was to determine differences among treatments for effectiveness in reduction of particular effects.

Materials and Methods

Animals

Nine female, crossbred, specific pathogen-free (SPF), weanling pigs, 20.6 ± 1.9 kg in weight, were individually housed in metabolism cages with water and a balanced corn-soybean meal diet available ad libitum. A routine analysis of the feed revealed no detectable aflatoxin, zearalenone, T-2 toxin, deoxynivalenol, diacetoxyscirpenol, or ochratoxin. The animals were acclimated to their environment for 5 days prior to dosing.

Experimental Procedure

Twenty-four hr prior to dosing, hair was removed from an area over the dorsal thoracolumbar region (approximately 20 x 15 cm) using electric clippers (Oster, model A-2 with #40 blade). Care was taken not to produce skin abrasions. This area was chosen since the back skin of weanling pigs approximates human forearm skin in its permeability characteristics (Marzulli et al., 1969).

The criterion for therapeutic efficacy was reduction of local cutaneous irritation. Therefore, dosing was based on application area rather than body weight. In a preliminary trial, a dose of 1.67 mg T-2/cm^2, similar to that used previously in topical T-2 studies in swine (Pang et al., 1987), resulted in a severe, rapidly occurring (24 hr) lesion. A reduced dose in subsequent trials resulted in a more acceptable post-treatment gradation in lesion severity. Thus, a dose of 0.67 mg T-2 toxin cm^2 was utilized in this study.

Prior to dosing, the dorsal thoracolumbar surface of each pig was washed thoroughly with water and soap (DVM Handsoap, Dermatologics for Veterinary Medicine, Inc., Miami, FL), rinsed thoroughly, and dried. Ten 3 x 3 cm dosing sites, 5 on each side of the midline and separated by at least 3 cm, were outlined on the pig's dorsal skin surface, caudal to the soapulae and cranial to the illial tuberosities. T-2 toxin (99% pure, produced in our lab) was dissolved in 90% dimethyl sulfoxide (DMSO, Aldrich Chemical Co., Milwaukee, WI) to a final concentration of 0.24 mg/ml. This concentration of DMSO was selected since the rate of diffusion of dissolved substances through the intact epidermis was shown to rise sharply as the concentration of DMSO approached 70%, reached a maximum at 90% and then decreased at higher concentrations (Kligman, 1965). The solution was distributed with a micropipette (Gilson Pipetman, Rainin Instrument Co., Woburn, MA) at a volume of 24.9 µl of dosing solution per site, which resulted in 6 mg of T-2 per dosing area.

Ten different treatments were randomly applied to individual sites on each pig including a T-2 positive control and a 90% DMSO vehicle control (Table I.16). Superactive charcoal (SuperChar-Vet, Gulf Biosystems, Dallas, TX) was combined with water in a 6:1 ratio, mixed into a paste, and applied so as to completely cover the dosing site. Preliminary studies had indicated that the superactive charcoal paste, when administered alone, had no apparent adverse effect on the skin. In treatments 3 and 4, this paste was left on the site until the time of necropsy. In treatments 5 to 8, the paste was removed by washing with soap placed on a moistened 3 x 3 cm gauze sponge. The technique was standardized and an attempt was made to completely wash the area without disseminating toxin to the surrounding skin surface. Treatments 9 and 10 consisted of the wash alone. All procedures were performed while the pigs were suspended in a canvas sling (Panepinto et al., 1983).

A modification of a previously described procedure for rats and rabbits was utilized to protect each dosing site (Aldrich et al., 1986). After each pig was treated, strips of hypoallergenic tape (Dermicell, Johnson and Johnson Products, Inc., New Brunswick, NJ) were placed between dosing sites to isolate them. Individual 5 x 5 cm squares of an air-permeable, water-impermeable 230 mesh monofilament polyester material (Advanced Process Supply, Chicago, IL) were then placed over the dosing sites and taped securely. This material allowed air access to the site while preventing loss of any remaining T-2 toxin or desquamated cellular debris as well as limiting surface contamination by foreign material. Finally, a piece of 28-gauge aluminum wire mesh screen, of sufficient size to cover all 10 dosing sites, was taped in place to further protect the area. Pigs were then returned to their cages and observed 3 times per day for the development of clinical abnormalities.

Lesion Evaluation: Gross and Microscopic

Three randomly selected animals were killed and necropsied at 1, 3, and 6 days postdosing since previous studies in our laboratory had shown that healing of T-2 induced skin lesions began by Day 7 (Page et al., 1987).

The animals were killed by electrocution and exsanguinated. The skin of the dosed areas along with adjacent, untreated skin and a small amount of subcutaneous fat were removed intact. Full-thickness sections (1 x 5 cm) of toxin-dosed and adjacent untreated skin were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, section at 6 µm, stained with hematoxylin and eosin, and examined by light microscopy.

Just prior to euthanasia, the treatment sites were graded blindly for lesion severity by 2 independent pathologists using a modification of the Draize test (Draize et al., 1944). The following criteria were graded (on a 0 to 4 basis) for each site. 1) area affected (percent involvement), 2) edema formation, 3) erythema, and 4) ulceration. Percent involvement of the dosed area was scored as follows: 0 = 0%, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = 76 to 100%.

Histologic sections of skin lesions were evaluated at 5 separate areas per section at 10x magnification, with every fourth field being evaluated. Both epidermal (including hair follicle) and dermal lesions were scored from 0 = none/minimal to 4 = very severe. The following 5 lesion criteria were scored for the epidermis: 1) inflammation, 2) necrosis, and 3) hyperplasia; and dermis: 4) edema and 5) inflammation; with a total maximum lesion score of 20.

Statistical Analysis

Due to the nature of the data which consisted of simultaneous measurements of p effects on one individual, and a primary emphasis on differences among effects, multivariate statistical methods were used for evaluation.

In statistical terms, each group of effects consists of p commensurable responses collected from individual sampling units (animals) according to k treatments or experimental conditions. The data from each treatment constitute a "profile" of that treatment's performance and certain hypotheses for the analysis of group or average profiles were tested. These hypotheses, 1) parallelism of the treatment mean profiles, 2) equality of treatment levels, and 3) equality of response means, were tested using multivariate repeated measures analysis of variance, as described by Morrison (1976). For each individual endpoint, treatment differences were determined using linear contrasts; $p < 0.10$ was considered significant.

In this study, 10 treatments were tested on each animal. For analysis, treatments were combined to form 3 groups, based on the therapy, irrespective of the time delay (which was not significant, $p > 0.10$). The groups were those where treatment consisted of: 1) superactivated charcoal alone (SAC), 2) a soap and water wash alone (SOAP), or 3) a combination of both techniques (SAC/SOAP). Prior to profile analysis, it was determined that all treatments were significantly different from the positive T-2 control. Therefore, controls were not included in the analysis since the primary objective was to compare therapeutic treatments with each other.

Results

Clinical Observations

No adverse systemic clinical effects were observed in any of the animals during the study.

T-2 toxin alone had an extremely deleterious effect on the skin (Table I.17). Marked reddening was observed on Day 1 which became progressively worse by Days 3 and 6. Similarly, edematous swelling was present on Day 1 and persisted through Day 6. Ulceration of the epidermal surface was slight by Day 3 and severe by Day 6. Sites where T-2 toxin application was followed by therapeutic intervention were similarly affected although the changes were less marked. DMSO alone had no effect on the application site.

Profile Analysis

Since we expected the profiles to change with time, separate analyses were carried out for each day. Representative profiles are shown in Figures I.29 and I.30. Excepting Day 3, overall treatment profiles for microscopic responses did not generally differ ($p \leq 0.10$), although certain endpoints within the profiles did differ significantly with treatment. With respect to gross pathologic response, the profiles on each day differed significantly ($p \leq 0.01$). Table I,17 summarizes treatment means for each endpoint. Table I.18 summarizes the significant contrasts for both gross and microscopic effects.

Gross Pathologic Comparison

With respect to percent involvement, edema, and erythema, mean lesion severity scores for SAC/SOAP combination treatments were significantly less

than those of SAC alone on Days 1, 3, and 6 post-treatment. A comparison of SAC with SOAP alone resulted in significantly lower mean scores for the latter treatment, with respect to percent involvement, edema, and erythema, on Days 1, 3, and 6 post-treatment. Mean lesion scores for SOAP versus SAC/SOAP combination treatments differed significantly for percent involvement, edema, and erythema on Day 1, ulceration on Day 3, and erythema on Day 6.

Ulceration was significantly more improved by application SAC than either SOAP or SAC/SOAP on Day 3, but the reverse was true on Day 6.

Histologic Comparison

With respect to epidermal hyperplasia and dermal edema, by Day 3 post-treatment both the SAC/SOAP and SOAP treatments were significantly more effective in reducing lesion severity than SAC alone. For dermal inflammation, SAC/SOAP was significantly more effective than SAC (contrasts), but the 3 treatments did not differ significantly overall (F test) for this lesion. Epidermal inflammation responded comparably to the 3 treatments. On Days 1 and 6 post-treatment, significant differences between treatments were less numerous, although they were more likely on Day 6 (Table I.18). The only significant difference between SOAP and SAC/SOAP treatments was on Day 1, where SAC/SOAP had a significantly lower mean dermal edema value.

Discussion

This study demonstrates that a superactive charcoal paste or a soap and water wash, when applied within 60 minutes after topical treatment of intact swine skin with T-2 toxin, significantly reduces the severity of resultant cutaneous lesion.

All therapeutic regimens were effective in reducing lesion severity resulting from T-2 toxin application. Significant differences in relative effectiveness were also seen between treatments. In each significant pair, the ordering of mean lesion severity was SAC/SOAP < SOAP < SAC. Therefore, when used as a single treatment, a soap and water wash appears to be more effective than application of a superactive charcoal paste in reducing the cutaneous effects of topical T-2 toxin.

These results do not support an additive therapeutic effect when both treatments were used sequentially. It was hypothesized that application of a superactive charcoal paste prior to washing would effectively bind the toxin, minimize interaction with the skin surface, prevent absorption, and thereby decrease the toxic effect when compared to soap and water alone. However, in this study a significant enhancement of therapeutic effect by SAC/SOAP combination therapy was only occasionally seen when compared to SOAP alone. It is possible that such an additive relationship might be manifested if the time was increased between toxin application and subsequent washing of the exposed site. Recent studies in rats reported the removal of 88.0 to 97.1% of topical [^{3}H] T-2 in DMSO by spraying with detergent and water 60 minutes postexposure. Only 49.3 to 65.8% was removed when spraying was delayed until 24 hr postexposure (Bunner et al., 1988).

Inspection of lesion severity scores of treatment combinations (treatments 5 to 8; Table I.16) suggested that the efficacy of those prococols were similar enough to preclude the recommendation of an optimum combination protocol. There were no statistical differences in evaluation parameters among the

combination treatments. As stated above, lengthening the time interval between the various treatment steps might result in a greater difference between treatment combinations.

Fairhurst et al. (1987) also recently examined the effectiveness of a soap solution in decreasing T-2 lesion severity. Doses of 0.5 and 500 μg T-2/cm^2 (compared to our 670 μg/cm^2) were applied to shaved dorsal skin of rats. They found soap and water washing to be very effective in reducing lesion severity at the lower dose with effectiveness decreasing as time between T-2 application and washing increased (up to 60 minutes). However, at the higher T-2 dose a soap solution was ineffective in reducing lesion severity. It is possible that swine skin responds differently than that of rats, but quantitative differences cannot be determined using their data since the method of quantitative analysis was not presented.

In preliminary studies, we observed only minor systemic clinical signs (hyperthermia, partial anorexia, and slight depression) in swine topically exposed to 43.5 to 44.8 mg/kg T-2 toxin in DMSO or acetone (unpublished data). T-2 toxin dissolved in ethyl acetate and applied topically (0.25 mg/animal) induced anorexia, lethargy, paresis, and death in rats (Bamburg et al., 1969). Similar topical application of T-2 toxin in DMSO resulted in death of 100% of dosed mice at 20, 30, and 40 mg/kg body weight (Schiefer and Hancock, 1984). Wannemacher et al. (1985) determined the LD_{50} of T-2 toxin in DMSO or methanol topically applied to guinea pigs to be 4 mg/kg. Other *in vitro* studies have indicated species differences in skin penetration by T-2 toxin. Human skin was shown to be far less permeable to T-2 toxin than that of guinea pigs or rats (Kemppainen et al., 1984; Kemppainen et al., 1987).

In previous studies with swine in our laboratory, after topical application of 15 mg/kg of T-2 in DMSO, neither free T-2 nor its metabolites were found in the plasma, bile, or urine, whereas significant quantities persisted in the skin and subcutaneous fat (Pang, 1987).

The results of this study and our previous studies suggest that swine skin may be less permeable to T-2 toxin than that of other common laboratory animals. It could thus serve as a more accurate model for predicting the effects on humans of topical T-2 exposure.

Additional studies are needed to further define the beneficial effects of an activated charcoal paste and soap and water in reducing local cutaneous damage after exposure to T-2 toxin. Various application times of superactive charcoal and/or soap and water may be examined to determine the relationship between treatment lag time and reduction of toxic effect.

Acknowledgements

The authors thank R. Manuel, B. Kindler, E. Owens, Drs. R. Lovell, and R. Poppenga for technical assistance, Dr. V. Pang for gross evaluation of skin slides, and S. Swanson for supplying the T-2 toxin.

These studies were supported in part by the U.S. Army Medical Research and Development Command, Contract No. DAMD 17-85-C-5224. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

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Table I.16 Treatments applied to individual dosing sites on each pig

Dosing Site	Treatment
1	90% DMSO
2	T-2 positive control
3	T-2 followed in 5 minutes by superactivated charcoal
4	T-2 followed in 60 minutes by superactivated charcoal
5	T-2 followed in 5 minutes by superactivated charcoal followed in 30 minutes by a soap and water wash
6	T-2 followed in 5 minutes by superactivated charcoal followed in 60 minutes by a soap and water wash
7	T-2 followed in 30 minutes by superactivated charcoal followed in 5 minutes by a soap and water wash
8	T-2 followed in 60 minutes by superactivated charcoal followed in 5 minutes by a soap and water wash
9	T-2 followed in 30 minutes by a soap and water wash
10	T-2 followed in 60 minutes by a soap and water wash

Table I.17 Treatment means for each lesion criteria

Day	Criteria[a]	Treatment Mean[b]						
		Histopathology			Gross Pathology			
		SAC	SAC/SOAP	SOAP	SAC	SAC/SOAP	SOAP	T-2 alone
1	1	0.167	0.333	0.500	3.667	1.083	1.833	4.00
1	2	0.000	0.000	0.000	1.833	0.500	0.833	2.00
1	3	0.333	0.417	0.500	2.500	1.250	1.917	3.00
1	4	0.167	0.083	0.500	0.000	0.000	0.000	0.00
1	5	1.000	0.500	0.833	---	---	---	---
3	1	1.167	0.500	0.833	2.833	1.250	1.333	4.00
3	2	0.833	0.000	0.333	1.833	0.708	0.583	2.70
3	3	2.167	0.917	1.000	2.833	1.500	1.917	3.80
3	4	1.167	0.333	0.500	0.000	0.042	0.333	0.80
3	5	1.667	0.750	0.833	---	---	---	---
6	1	0.833	0.750	0.500	3.000	1.417	1.917	4.000
6	2	0.000	0.083	0.167	1.000	0.458	0.083	3.000
6	3	1.333	1.000	0.667	1.833	0.708	0.167	2.200
6	4	1.000	0.667	0.333	2.000	1.083	1.000	4.000
6	5	1.333	0.833	0.500	---	---	---	---

[a]Criteria for histopathology are: 1 = epidermal inflammation, 2 = epidermal necrosis, 3 = epidermal hyperplasia, 4 = dermal edema, 5 = dermal inflammation.

Criteria for gross pathology are: 1 = percent involvement, 2 = edema, 3 = erythema, 4 = ulceration.

[b]SAC = Treatments 3 and 4.
SAC/SOAP = Treatments 5 to 8.
SOAP = Treatments 9 and 10.

Table I.18 Significant contracts between treatment pairs with respect to individual lesion criteria

Criteria[a]								
Gross Pathology	Histo-pathology	Treatment[b] Comparison	Histopathology[c] Day 1	Day 3	Day 6	Gross Pathology[c] Day 1	Day 3	Day 6
Percent Involvement	EI	1 vs. 2	---	---	---	****	****	****
		1 vs. 3	---	---	---	****	***	**
		2 vs. 3	---	---	---	***	---	---
Edema	EN	1 vs. 2	---	**	---	****	****	---
		1 vs. 3	---	---	---	****	****	**
		2 vs. 3	---	---	---	*	---	---
Erythema	EH	1 vs. 2	---	***	---	****	***	****
		1 vs. 3	---	**	**	**	*	****
		2 vs. 3	---	---	---	***	---	*
Ulceration	DE	1 vs. 2	---	***	---	---	---	****
		1 vs. 3	---	**	*	---	***	****
		2 vs. 3	**	---	---	---	***	---
	DI	1 vs. 2	*	*	---			
		1 vs. 3	---	---	**			
		2 vs. 3	---	---	---			

[a]Criteria for histopathology are: epidermal inflammation, epidermal necrosis, epidermal hyperplasia, dermal edema, dermal inflammation.

[b]1 = SAC (Treatments 3 and 4).
2 = SAC/SOAP (Treatments 5 to 8).
3 = SOAP (Treatment 9 and 10).

[c] *$p \leq 0.10$
**$p \leq 0.05$
***$p \leq 0.01$
****$p \leq 0.001$

Figure I.29 Profiles of mean scores for gross pathology assessment criteria for each treatment group by day.

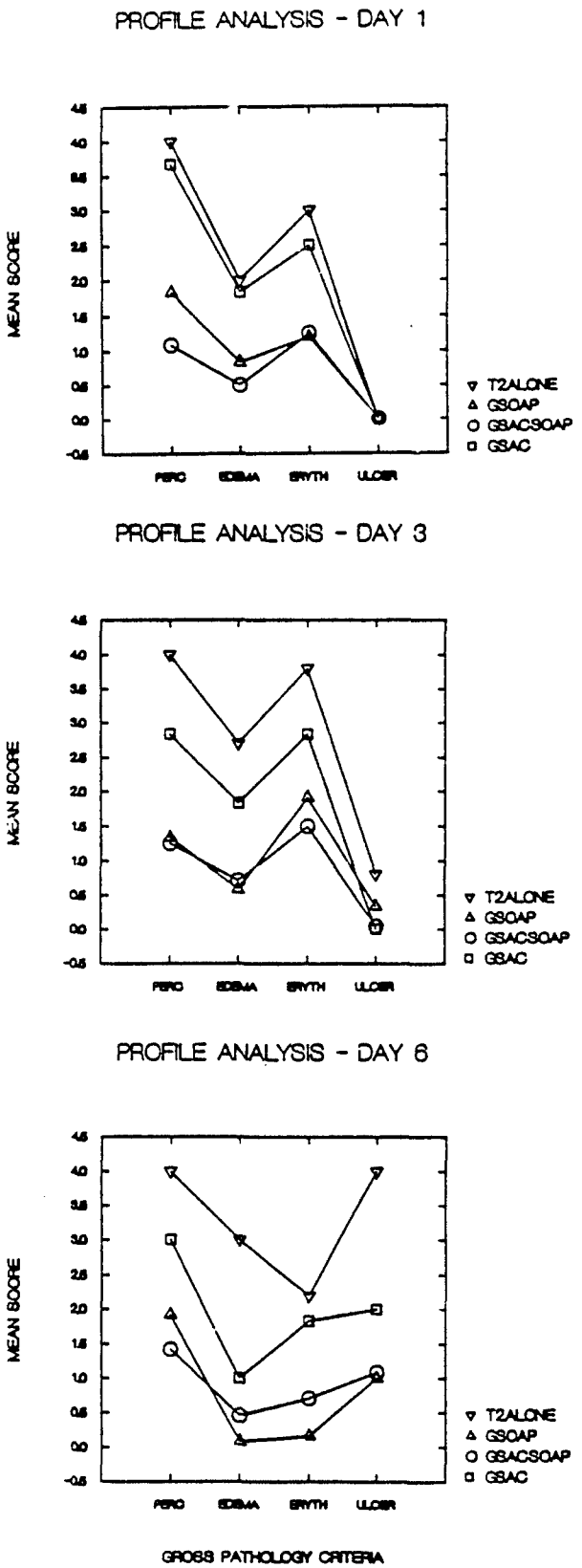
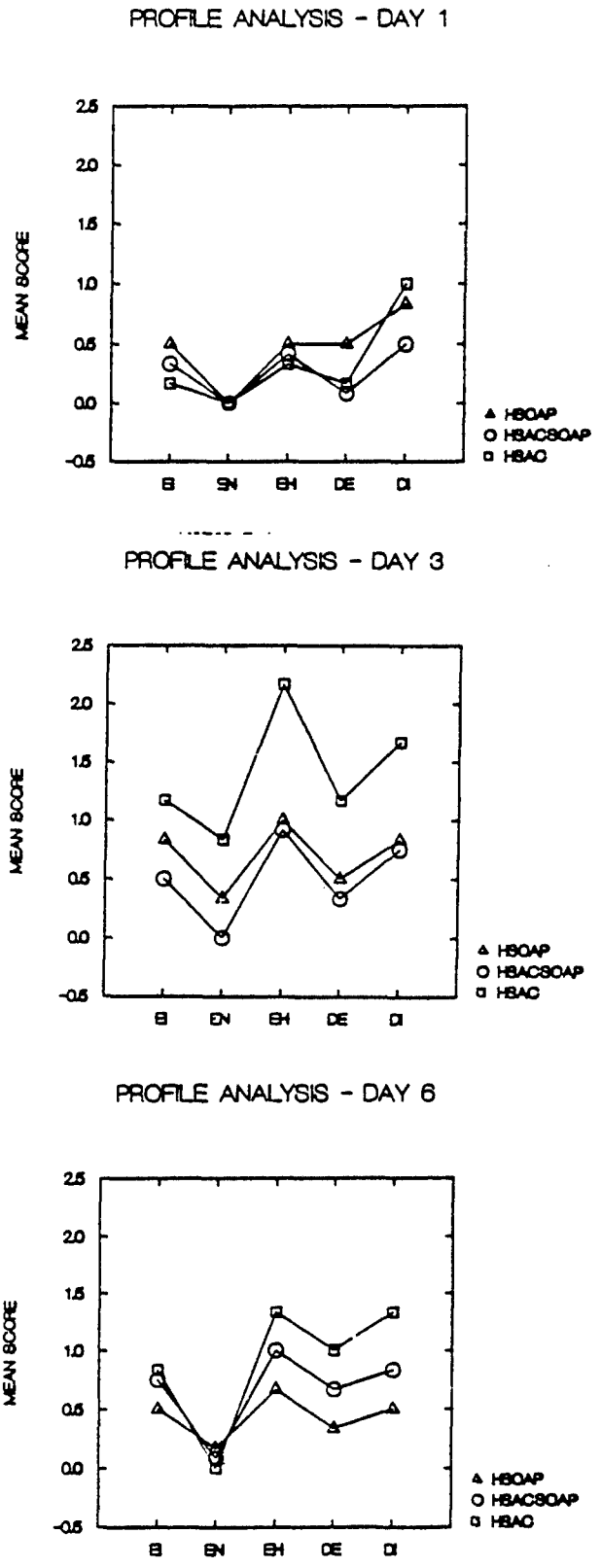


Figure I.30 Profiles of mean scores for histopathology assessment criteria for each treatment group by day.



H. Assessment of a General Therapeutic Protocol for the Treatment of Acute T-2 Toxicosis in Swine

by
Robert H. Poppenga, Val R. Beasley, and William B. Buck

Abstract

T-2 toxin, a trichothecene mycotoxin suspected of being used as a chemical warfare agent, was administered intravenously to swine at a dose of 3.6 mg/kg body weight (iv LD_{50} approximately 1.2 mg/kg). Four different therapeutic protocols were assessed for their efficacy in the treatment of the resultant acute T-2 toxicosis syndrome. One therapeutic protocol included the combined use of metoclopramide, activated charcoal, magnesium sulfate, dexamethasone sodium phosphate, sodium bicarbonate, and normal saline (all therapy). The other 3 protocols utilized the same agents less 1 of the following: sodium bicarbonate, normal saline, or the combination of activated charcoal and magnesium sulfate. All 4 treatment groups had improved survival times compared to a positive T-2 control group. Within the limits of the study, it would appear that the removal of activated charcoal and magnesium sulfate was most detrimental to the T-2 toxin dosed swine.

Introduction

T-2 toxin is a secondary fungal metabolite produced by various species of Fusarium. It belongs to a large group of mycotoxins called the trichothecenes. T-2 toxin, in combination with other mycotoxins, was allegedly used as a chemical warfare agent in Southeast Asia and Afghanistan (1). It is an especially toxic compound with a reported intravenous LD_{50} for swine of approximately 1.2 mg/kg (2).

In experimental animals acutely exposed to T-2 toxin by the oral, intravenous, or inhalation routes, a variety of organ systems were affected, especially those with actively dividing cells such as the lymphoid tissues, gastrointestinal tract, and bone marrow (3-5). Acute exposure to sufficient T-2 toxin resulted in the rapid onset of circulatory shock characterized by reduced cardiac output, profound arterial hypotension, and lactic acidosis (6). T-2 toxin is a potent emetic agent with severe and prolonged emesis beginning within 15 to 30 min after toxin administration. Death is due to circulatory collapse.

T-2 toxin is rapidly metabolized by the liver. Significant amounts of T-2 toxin and its metabolites are eliminated in the bile as glucuronide conjugates (8). The possibility exists that the glucuronides are deconjugated in the intestinal tract by microbial action and undergo enterohepatic recirculation. This may contribute to the shock syndrome following acute T-2 toxin exposure.

Several drug agents have shown some efficacy in alleviating acute T-2 toxicosis. Bratich (9) demonstrated the effectiveness of super-activated charcoal for the treatment of T-2 toxicosis in rats. Dexamethasone sodium phosphate improved short-term survival in mice (10) and rats (11) given otherwise lethal doses of T-2 toxin. Similarly, methylprednisolone sodium succinate provided an increased rate of survival in T-2 toxin treated rats (12).

With these studies in mind, we elected to include a glucocorticosteriod in each therapeutic regimen tested. Because of the circulatory shock and acidosis which accompany acute T-2 toxicosis, it was hypothesized that other supportive measures such as maintenance of arterial blood pressure using normal saline and correction of acidosis with sodium bicarbonate might further improve survival. The effects of dexamethasone sodium phosphate in combination with other supportive measures were therefore assessed in young, female swine acutely exposed to a lethal dose of T-2 toxin.

Materials and Methods

Approximately 3 to 4 weeks prior to inclusion in this study, 25 kg, female, cross-bred swine were obtained from a nearby commercial swine operation. Three to 4 animals were randomly assigned to each of the 5 experimental groups listed in Table I.19. After acclimation, general anesthesia was induced with halothane (Fluothane, Fort Dodge Laboratories, Fort Dodge, IA) and saline-filled, Tygon (A. Daigger Co., Chicago, IL) catheters were surgically implanted into the left atrium, pulmonary artery, and internal carotid artery via a left lateral thoracotomy. The internal carotid catheter was advanced into the ascending aorta. The catheters were heparinized to maintain patency, threaded through the left thoracic wall and buried subcutaneously. A 3-week recovery period was allowed following surgery.

Twelve hr prior to dosing, the previously buried catheters were exteriorized following induction of general anesthesia with halothane. On the morning of dosing, individual animals were placed in a lateral squeeze cage which allowed a limited degree of free movement. Phasic and mean pressure in the aorta, pulmonary artery, and left atrium were recorded on a multichannel physiograph (Gilson Medical Electronics, Inc., Middleton, WI) using noncompliant, fluid-filled systems and pressure transducers (P23D, Statham Medical Instruments, Gould, Inc., Oxnard, CA). T-2 toxin was administered iv at a dose of 3.6 mg/kg body weight in a 50 percent ethanol solution over 2 to 3 min and therapeutic protocols begun. A maximum observation period of 48 hr was selected after which surviving animals were anesthetized with a barbiturate and exsanguinated. The 48 hr survival times were considered to be censored since the true survival times could not be determined due to the scheduled euthanasia.

The individual therapeutic agents utilized in this study and their administration protocols are given in Table I.20. Doses employed were either based on previously recognized uses of the particular agent and were thus the same for each individual on a mg/kg body weight basis or were based on an attempt to maintain certain physiologic parameters such as arterial blood pressure or arterial blood pH within normal limits. In the latter case, the amounts administered varied among individual animals depending on their particular needs. Since T-2 toxin is a potent emetic, metoclopramide was included in each of the therapeutic protocols, including the positive controls, to assess its efficacy as an antiemetic and, at the same time, to facilitate retention of the activated charcoal/magnesium sulfate combination given to groups 2, 4, and 5.

A k-sample test capable of handling $k > 2$ with censored observations was employed to detect an overall significant difference in survival time among the 5 groups (13). Since only improved survival was hypothesized, a 1-tailed test of significance was employed. Pairwise comparisons of survival times between each treatment group and the control group were assessed utilizing a modification of Gehan's generalized Wilcoxon test (13).

Results

Survival times for individual animals and group mean survival times are given in Table I.19. Mean survival times were not calculated for groups in which 1 or more animals survived the full 48 hr observation period. The P values for the 1-tailed tests of significance for comparison of treatment groups to the control group were .030, .040, .026, and .030 for groups 2, 3, 4, and 5, respectively.

Substantial variation in time to death for individual animals within a particular treatment group did occur. For example, survival times for treatment group 2 ranged from 9.5 to 48 hr. The survival times for the T-2 control group were remarkably consistent, however, ranging from 7.8 to 9.4 hr.

Discussion

While the number of animals per group was small, certain observations are worth noting. Two of the 3 animals given the complete array of therapeutic agents survived for the full 48 hr observation period. Had the data not been censored because of the scheduled euthanasia, a greater difference in survival time compared to the control group would have been expected. The same would be true for treatment groups 4 and 5. All animals which survived to 48 hr were alert and active despite having been given a dose of T-2 toxin 3 times higher than an expected LD_{50}. In many previous studies using lower doses of T-2 toxin, given by various routes of administration, we have found that swine surviving 36 hr post-toxin exposure are generally exhibiting a substantial reduction in clinical signs and would appear likely to make a full recovery.

There were no clear-cut differences in survival times for those pigs given all therapy (group 2), all therapy without sodium bicarbonate (group 4), or all therapy without saline (group 5). The inclusion of sodium bicarbonate was helpful in maintaining a more normal arterial blood pH. The intravenous administration of a large volume of normal saline, however, was not effective in maintaining mean aortic blood pressure above 65 mmHg. The effect of the different therapeutic protocols on various physiologic parameters will be discussed more thoroughly in a subsequent paper.

Interestingly, the group given no activated charcoal or magnesium sulfate did not appear to do as well as the other 3 treatment groups. We did not anticipate that orally administered activated charcoal would have any beneficial effect following parenteral administration of T-2 toxin. Two hypotheses may account for this apparent benefit. A significant portion of an intravenously administered dose of T-2 toxin is eliminated in the bile as glucuronide conjugates (8). It is possible that intestinal microbial action may cause deconjugation and result in substantial enterohepatic recirculation of T-2 toxin or its metabolites, thus prolonging systemic effects of the toxin. Activated charcoal may bind the toxin within the intestinal tract preventing its reabsorption. Alternatively, activated charcoal may aid in the binding of endotoxin elaborated by intestinal microflora. Since the intestinal mucosa is severely damaged in acute T-2 toxicosis, endotoxin may enter the systemic circulation unhindered by normal barrier mechanisms. Activated charcoal is known to be effective in adsorbing endotoxin (14).

Only partial success was achieved with the use of the metoclopramide as an antiemetic. This may have been due to an inappropriate dosage regimen and

further evaluation, particularly of a continuous intravenous infusion, may be warranted.

Combined general supportive therapies do appear to enhance survival in swine given an otherwise lethal dose of T-2 toxin. More specific therapies may emerge as knowledge concerning the cellular pathophysiology of T-2 toxin improves.

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Table I.19 Swine therapeutic study: experimental groups and survival data

Group (n = 3)*	Treatment	Mean Weight (kg)	Survival Time (Hours)	Mean Survival Time (Hours)
1	Control T-2 + Metoclopramide	51.0	9.4, 7.8, 8.6	8.6
2	Metoclopramide Dexamethasone Normal saline and $NaHCO_3$ Activated charcoal + $MgSO_4$ (All therapy)	53.3	9.5, 48.0, 48.0	+
3	Metoclopramide Dexamethasone Normal saline and $NaHCO_3$ (No activated charcoal or $MgSO_4$)	45.5	15.8, 20.1, 18.0	18.0
4	Metoclopramide Dexamethasone Activated charcoal + $MgSO_4$ Normal saline (No $NaHCO_3$)	47.8	11.3, 22.5, 30.0, 48.0	+
5	Metoclopramide Dexamethasone Activated charocal + $MgSO_4$ $NaHCO_3$ (No saline)	53.6	23.5, 48.0, 48.0	+

*Treatment group 4: n = 4.
+No group means were calculated due to presence of censored observations.

Table I.20 Swine therapeutic study: drug administration protocol

Drug	Source	Dosage Regimen
Metoclopramide	Injectable form (5 mg/ml) courtesy of A. H. Robins Company.	1 mg/kg body weight iv immediately prior to T-2 toxin administration and 1/4 and 1-1/4 hr post-T-2 toxin.
Activated Charcoal	SuperChar● courtesy of Gulf BioSystems, Inc.	2 grams activated charcoal (dry weight) per kg body weight in 420 ml tap water po 1/2 hr and 4 hr post-T-2.
Magnesium Sulfate	Epsom salt, magnesium sulfate USP, purchased from Dow Chemical Company.	1/2 gram/kg body weight po mixed with activated charcoal slurry and administered 1/2 hr and 4 hr post-T-2.
Dexamethasone Sodium Phosphate	Azium S/P● (4 mg dexamethasone sodium phosphate/ml), purchased from Schering Corporation.	6 mg/kg body weight iv immediately and 4 hr post-T-2, then 4 mg/kg 8 and 12 hr post-T-2, followed by 2 mg/kg 16 and 20 hr post-T-2 and 1 mg/kg 24 hr post-T-2.
Sodium Bicarbonate	5 percent sodium bicarbonate injection, USP. Purchased from Abbott Laboratories.	Variable speed iv drip based on hourly arterial blood pH measurements. Started if pH < 7.350 and stopped if pH > 7.350.
Normal Saline	0.9 percent sodium chloride injection, USP. Purchased from Abbott Laboratories.	Rapid iv drip (gravity flow) as MAP* begins decline. Administration slowed to maintenance levels if MAP does not respond or if CVP+ > 10 mmHg.

*Mean arterial blood pressure.
+Central venous pressure.

I. The Effect of Therapeutic Intervention on the Pathophysiology of Acute T-2 Toxicosis in Intravenously Dosed Swine

by
Robert H. Poppenga, Gregg R. Lundeen, Val R. Beasley, and William B. Buck

Abstract

Four therapeutic protocols utilizing different combinations of dexamethasone sodium phosphate (DEX), normal saline (SAL), sodium bicarbonate (BICARB), and superactivated charcoal (SAC) + magnesium sulfate (MS) were evaluated for efficacy in swine given an acutely lethal dose (3.6 mg/kg) of T-2 toxin iv. A number of physiologic parameters known to be affected by T-2 toxin were measured including hemodynamic, blood-gas, hematologic, and clinical chemistry variables. There were no clearcut effects of therapy on hemodynamic variables. The iv administration of normal saline did not maintain aortic mean pressure but did appear to result in a relative degree of hemodilution, an increase in urine production, and amelioration of elevations in serum concentrations of potassium, phosphorus, and creatinine. The iv administration of sodium bicarbonate lessened the decline in arterial blood pH. Superactivated charcoal (SAC), given orally, improved survival but did not appear to have a significant effect on measured parameters. The oral administration of magnesium sulfate caused an increase in serum magnesium concentrations. The antiemetic, metoclopramide, as given to all swine in the study, did not prevent emesis induced by T-2 toxin.

Introduction

T-2 toxin is a secondary fungal metabolite produced by several species of Fusarium. It belongs to a large group of sesquiterpene mycotoxins called trichothecenes. The natural occurrence of T-2 toxin has been associated with several human and animal diseases including alimentary toxic aleukia (ATA) of man in the Soviet Union (Joffe, 1971), moldy corn toxicosis of dairy cattle in the United States (Hsu et al., 1972), akakabibyo or red-mold disease of man and livestock in Japan (Saito and Ohtsubo, 1974), and bean-hull poisoning of horses in Japan (Ueno et al., 1972). More recently, T-2 toxin has been implicated as a component of the chemical warfare agent "yellow rain" (Mirocha et al., 1983; Rosen and Rosen, 1983).

The pathophysiology of acute T-2 toxicosis has been studied in a number of experimental animal species (Sato et al., 1975; DeNicola et al., 1978; Weaver et al., 1978; Feuerstein et al., 1985; Lorenzana et al., 1985a,b; Lundeen et al., 1986; Beasley et al., 1987). Administration of lethal doses of T-2 toxin causes circulatory shock characterized by hemodynamic alterations such as declines in cardiac output and mean arterial blood pressure; lactic acidosis; elevations of plasma catecholamine, prostaglandin, and renin concentrations; and changes in organ blood flows (Lorenzana et al., 1985a, Feuerstein et al., 1985; Lundeen et al., 1986; Siren et al., 1986; Beasley et al., 1987). Alterations in hematologic, blood-gas, and serum chemistry parameters also occur but are more variable both within a given species and between species (Chan and Gentry, 1984; Weaver et al., 1978; Lorenzana et al., 1985a,b; Feuerstein et al., 1985).

In addition to the above alterations, characteristic histopathologic changes occur (DeNicola et al., 1978; Weaver et al., 1978; Pang et al., 1987). At highly toxic doses, consistent histologic changes include severe lymphoiysis in lymphoid tissues such as lymph nodes, thymus, and spleen and severe

congestion and necrosis of the gastrointestinal mucosa, particularly the glandular portion of the stomach and the small intestine.

The most efficacious therapeutic protocols for the treatment of acute T-2 toxicosis as determined by survival data have included iv or ip administration of high doses of water-soluble salts of glucocorticosteroids, oral administration of superactivated charcoal (SAC), or a combination therapy including glucocorticosteroids, SAC plus a saline cathartic and supportive care (Fricke, 1985; Tremel et al., 1985; Galey et al., 1987; Poppenga et al., 1987a,b). However, the effect of therapeutic intervention on pathophysiologic alterations has not been reported.

The goal of the present study was to use swine given an otherwise lethal dose of T-2 toxin iv to determine the effect of therapeutic intervention on a number of physiologic parameters known to be altered in acute T-2 toxicosis. The ability of the different therapeutic protocols used to improve survival has been reported elsewhere (Poppenga et al., 1987a).

Materials and Methods

Animals

White, female, crossbred swine,[1] weighing between 38 and 69 kg, were injected with erysipelas bacterin[2] and acclimated to the large animal holding facility at the College of Veterinary Medicine, University of Illinois. The swine were fed a 16% protein, ground corn/soybean meal ration. All batches of feed offered to the experimental animals were free from detectable concentrations of deoxynivalenol, diacetoxyscirpenol, zearalenone, T-2 toxin, and aflatoxins B_1, B_2, G_1, and G_2.[3]

Toxin

The T-2 toxin used in this study was prepared in our laboratory from extracts of *Fusarium sporotrichiodes* grown on rice culture. The purity of the toxin was demonstrated to be > 95% by gas chromatography with flame ionization detection[4] of its trimethylsilylether derivative.

Surgical Preparation of Animals

Four to 6 weeks prior to the inclusion of individual animals in the study, anesthesia was induced by the administration of 5% halothane[5] in oxygen using a nose cone. After endotracheal intubation, anesthesia was maintained with 0.5 to 1.0% halothane in oxygen using a closed-circuit system[6] and positive pressure ventilation.[7] The heart rate and rhythm were monitored using a heart monitor.[8] A left lateral thoracotomy followed by a percardiotomy was performed to expose the left atrium and base of the pulmonary artery. The internal thoracic artery was also exposed at this time. Saline-filled Tygon catheters[9] were implanted in the pulmonary artery and left atrium. Another catheter was placed in the ascending aorta via the internal thoracic artery. The ends of the catheters were tunneled through the left lateral thoracic wall near the thoracotomy incision and fitted with injection caps. The catheters were next filled with heparin and the ends buried sc. A fourth catheter was implanted in the anterior vena cava via the left jugular vein in a manner similar to the others. All skin incisions were closed with polyglycolic acid[10] sutures which were removed 7 to 10 days after the surgery.

Experimental Protocol

The individual swine were randomly assigned to either a positive control group or 1 of 4 treatment groups (Table I.21). Since only 1 animal could be monitored at a time, 3 replicates of each series of 5 treatments were conducted.

Approximately 16 hr prior to inclusion in the study, the swine were anesthetized with halothane in a mixture with oxygen. The ends of the sc buried catheters were located and exteriorized through small skin incisions. Their patency was checked and fresh heparin instilled. Two 18 ga, indwelling catheters[11] were placed in ear veins and sutured to the skin. A bandage was placed over each catheter for protection. A foley catheter[12] was placed in the urinary bladder via the urethra and sutured in place. The swine were allowed to recover from anesthesia and held in a small pen overnight. The swine were fasted for 12 hr prior to dosing with T-2 toxin, although water was available ad libitum.

On the day of the study, each pig was placed in an adjustable restraining cage which allowed for a limited degree of free movement. The animals were able to stand or lie down as they wished. All swine appeared to rapidly adapt to this method of restraint.

Prior to T-2 toxin administration, 2 baseline readings, 1 hr apart, were obtained for each parameter of interest. The first dose of metoclopramide, which was given to all swine, was next infused via an ear vein over a 10 min period using an infusion pump.[13] T-2 toxin was then administered at 3.6 mg/kg in a 50% ethanol:50% saline mixture (0.1 ml vehicle/kg) via the anterior vena cava. The total dose of T-2 toxin was given over a 2 to 3 min infusion. Therapeutic regimens were instituted immediately according to a predetermined protocol which is outlined in Table I.22. Measurements were obtained each hr for the first 8 hr after toxin administration. During sampling periods, all catheters were frequently flushed with heparinized saline to ensure patency and prevent blood clot formation. During the course of the experiment, each pig was given 4 to 6 liters of fluid from this flushing.

Those animals surviving the 48 hr observation period were anesthetized with sodium thiamylal and exsanguinated. A complete post-mortem examination was conducted immediately thereafter.

Hemodynamic Measurements

Phasic and mean pressures in the aorta, pulmonary artery, left atrium, and anterior vena cava were recorded on a multichannel physiograph[14] using non-compliant, fluid-filled systems and pressure transducers.[15] The transducers were zeroed at the level of the scapulohumeral joint which was considered to correspond to the level of the right atrium.

Cardiac output was determined, in triplicate, using a dye-dilution technique (Manohar et al., 1978). Indocyanine green USP[16] was injected into the left atrium and blood was withdrawn from the aorta at a known constant rate through a linear densitometer.[17] The resulting curve was determined by semilogarithmic plotting of the downslope on a computer.[18]

Total peripheral resistance (TPR) was calculated as the quotient of aortic mean pressure (AOM) in mmHg divided by the cardiac index (CI) expressed as

ml x min^{-1} x kg^{-1}. Pulmonary vascular resistance (PVR) was calculated by subtracting the left atrial mean pressure (LAM) in mmHg from the pulmonary artery mean pressure (PAM) in mmHg and dividing the difference by the CI. Left and right ventricular work (LVW and RVW, respectively) expressed as kg x mmHg x min^{-1}/kg bw was calculated from the formula: CI x either AOM or PAM x 1.36 x 10^{-2} where 1.36 is the conversion factor for changing pressure from mmHg to g/cm^2. Stroke volume (SV) was determined by dividing the CI by the heart rate (HR).

Blood-Gas and Related Measurements

Arterial blood was anaerobically collected from the aortic catheter for determination of arterial blood gas tensions and pH (PaO_2, $PaCO_2$, and pH_a). PaO_2, $PaCO_2$, and pH_a were corrected to the rectal temperature of the animal using nomograms built into the blood-gas analyzer[19] (Kelman, 1966; Kelman and Nunn, 1966; Severinghaus, 1966; Burnett and Noonan, 1974). Calibration of the blood-gas analyzer was checked after each sampling period using swine blood tonometered[20] at 38°C with gases of known oxygen and carbon dioxide tensions.

Hematologic and Clinical Chemistry Measurements

All blood samples were collected through the aortic catheter. Blood was collected into siliconized tubes[21] and allowed to clot at 37°C for 2 hr. The samples were then centrifuged and the serum was collected for biochemical and enzymatic measurements. The concentrations or activities of creatinine (CREA), total protein (TP), phosphorus (PHOS), alkaline phosphatase (AP), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), total calcium (TOTCAL), blood urea nitrogen (BUN), sodium (NA), potassium (K), chloride (CL), aspartate aminotransferase (AST), cholesterol (CHOL), bilirubin (BILI), albumin (ALB), and globulin (GLOB) were determined by an autoanalyzer.[22] When the determined values were above the working range of the autoanalyzer, the serum samples were diluted and reanalyzed. An aliquot of serum (4 ml) was placed in a membrane cone[23] and centrifuged at 1000 xg for 20 min. The calcium concentration of the ultrafiltrate (serum ionized calcium) was then determined by the autoanalyzer. Serum magnesium (MG) concentrations were determined by atomic absorption spectrophotometry.[24] A separate blood sample was placed in a chilled tube containing sodium fluoride.[25] The sample was spun for 10 min at 2000 xg in a refrigerated centrifuge and the plasma separated. The plasma was analyzed for glucose (GLU) concentration by autoanalyzer.

Whole blood was collected for hematologic examination in tubes containing EDTA.[26] Hemoglobin (HB) content was determined by the cyanmethemoglobin method.[27] Leukocyte and erythrocyte counts were determined by an electronic particle counter.[28] The white blood cell differential was determined from blood smears. One hundred cells were observed for every 1 x 10^4 white blood cells per µl counted by the electronic counter.

For plasma lactic acid (LA) determination, blood was collected into chilled syringes containing chilled perchloric acid. The blood was then vortexed for 30 sec, the protein precipitate was removed by centrifugation, and the plasma was placed in polyethylene tubes and stored at 4°C. Analysis was done within 7 days of collection using a commercially available kit.[29]

Statistical Analyses

Using a computerized program,[30] Pillais' Trace transformed to a F-statistic was used to evaluate the overall effect of treatment and time. Where significant overall treatment effects were noted, orthogonal contrasts were used to detect significant differences. The specified contrasts for each parameter of interest were based on a priori hypotheses concerning the beneficial effect of a particular therapeutic agent. Due to the small sample sizes, a level of significance of $\alpha = 0.10$ was chosen for all analyses. Data ± SEM for all parameters are reported in Appendix I.B.

Results

Hemodynamic Measurements

There was a significant difference among the various groups across all time points with regard to HR and LAM ($p = 0.007$ and 0.044, respectively). There was a significant overall change in HR, CI (Fig. I.31), AOM (Fig. I.32), and LVW with regard to time ($p = 0.013$, 0.033, 0.0001, and 0.073, respectively). Over time, the HR for all the experimental groups increased, with a greater increase noted for the positive control group and the group given all therapy minus SAC + MS. There was a significant difference in HR between the group given all therapy minus SAC + MS compared to the remaining treatment groups taken together ($p = 0.0006$), but not between the positive control group and the same 3 treatment groups. LAM declined for each group through the first 2 hr. For the positive control group, the decline continued through 3 hr and then stabilized. For the treatment groups the declines in LAM reversed themselves by 3 hr and then stabilized or gradually increased through 8 hr. The CI initially increased for all groups except the group given all therapy minus SAL. There was a subsequent decline in the positive control group and the group given all therapy minus SAL, while the CI remained somewhat stable in the other 3 groups during the 8 hr observation period with the exception of a sudden drop between 7 and 8 hr in the group given all therapy. Overall, the positive control group displayed the most prolonged and severe decline, despite the absence of a statistically significant difference. AOM and LVW gradually declined and then stabilized in a similar manner for all groups.

Blood-Gas and Related Measurements

There was a significant difference among the various groups across all time points for PaO_2, $PaCO_2$, pHa (Fig. I.33), arterial bicarbonate ($paHCO_3$), LA (Fig. I.34), and body temperature (BT) ($p = 0.002$, 0.031, 0.018, 0.003, 0.065, and 0.015, respectively). Across all treatments, there was a significant difference among time points for pHa, LA, and BT ($p = 0.001$, 0.007, and 0.05, respectively). While there was an initial decline in pHa for all groups, the pHa began to reverse itself in the treatment groups by 4 to 6 hr. However, there was a subsequent decline in pHa between 7 and 8 hr in the group given all therapy. In the positive control group, the pHa continued to decline with a sudden decrease just prior to death (mean time to death of 8.5 hr). There was a significant treatment difference between the positive control group and the group given all therapy ($p = 0.071$). In addition, there was a significant treatment difference between the group given all therapy minus BICARB and the other treatment groups taken together ($p = 0.026$).

Lactic acid concentrations were similar for all groups between 1 and 3 hr after toxin administration. At 4 hr there was an increase in LA

concentration for the positive control group which continued over the next 4 hr, with a large rise noted just prior to death. The LA concentrations remained stable in the treatment groups through 7 hr at which time there was a sudden rise in LA noted for the groups given all therapy and all therapy minus SAC + MS. There was a significant treatment difference between the positive control group and the group given all therapy ($p = 0.006$).

Body temperatures were similar among all experimental groups through the first 3 hr after toxin administration. Thereafter, the body temperatures of the positive control group gradually increased over the next 5 hr and decreased in the treatment groups.

There were statistically significant differences for PaO_2, $PaCO_2$, and HCO_3 between the group given all therapy minus BICARB and the other treatment groups taken as a whole ($p = 0.006$, 0.012, and 0.008, respectively). In the positive control group and the group given all therapy minus HCO_3, PaO_2 began to rise and $PaCO_2$ began to decline by 4 hr after toxin administration. In the other treatment groups, $PaCO_2$ declined by 3 hr after toxin administration and then slightly increased or remained steady through 8 hr (with the exception of a sudden decline between 7 and 8 hr in the group given all therapy). In the same 3 treatment groups, $PaCO_2$ decreased slightly or remained stable over the 8 hr observation period. The arterial HCO_3 concentrations declined for all groups through 3 hr. Thereafter, the decline continued in the positive control group and the group given all therapy minus BICARB, while concentrations stabilized in the remaining treatment groups.

Hematologic Measurements

There was a significant difference among the various groups across all time points with regard to red blood cell count (RBC), HB, and hematocrit (HCT) (Fig. I.35) ($p = 0.029$, 0.015, and 0.027, respectively). Across all treatments, there was a significant difference among times of observation for RBC, white blood cell count (WBC), HB, nucleated red blood cell count (NRBC), HCT, absolute segmented neutrophil count (ABSEG), and absolute lymphocyte count (ABLYM) ($p = 0.003$, 0.001, C .001, 0.024, 0.001, 0.0001, and 0.004, respectively). The HCT initially increased for all groups following toxin administration. A decline was then noted for all treatment groups with the highest values consistently observed in the positive control group. There was a significant difference between the positive control group and the group given all therapy ($p = 0.016$) but no difference between the group given all therapy minus SAL and the positive control group or the other 3 treatment groups taken together. In general, the HCT, RBC, and HB values followed the same temporal pattern. As for HCT, there were sudden, initial elevations in all the experimental groups for RBC and HB by 1 hr following toxin administration, with subsequent gradual declines for all the treatment groups and stable or increased values noted in the positive control group. Initial increases in ABSEG and ABLYM numbers were noted by 1 hr followed by subsequent declines at 2, 3, and 4 hr. Thereafter, ABSEG numbers began to increase for all groups by 5 hr, whereas the ABLYM numbers continued to decline through 8 hr. The WBC numbers reflected the changes noted for ABSEG and ABLYM. The NRBC numbers began to increase for all experimental groups by 3 hr after toxin administration and continued to increase through 8 hr.

Clinical Chemistry Measurements

There was a significant difference among the various groups across all time points for serum Mg (Fig. I.36), K, PHOS, TP (Fig. I.37), ALB, GLOB, and

CREA. Across all treatments, there was a significant difference among time points for CREA, TP, PHOS, AP, ALT, LDH, TOTCAL (Fig. I.38), BUN, NA, K, CL, AST, GLUC (Fig. I.39), CHOL, and ALB. At 5 hr there was a dramatic increase in serum Mg for all groups except the group given all therapy minus SAC + MS. The group given all therapy minus SAC + MS had significantly ($p = 0.047$) lower serum Mg values when compared to the positive control group. In addition, the serum Mg of the group given all therapy minus SAC + MS was significantly ($p = 0.008$) less than the remaining treatment groups taken as a whole.

Total serum protein tended to decline for all groups over time. However, there was a significant difference between the positive control group and the group given all therapy ($p = 0.002$). Also, the group given all therapy minus SAL had significantly higher serum protein values than remaining treatment groups taken together ($p = 0.003$). There were declines in TOTCAL and plasma GLU concentrations over time for all groups with no significant differences noted among the groups despite the spike in glucose observed in the positive control group at 2 and 3 hr postdosing. There were gradual increases over time for CREA, PHOS, AP, ALT, LDH, BUN, NA, K, CL, and AST for all treatment groups, with the elevations in CREA, PHOS, ALT, and BUN being more pronounced in the positive control group compared to the treatment groups. Significantly higher concentrations of PHOS and CREA but not BUN were noted in the positive control group when compared to the group given all therapy. When the group given all therapy minus SAL was compared to the other 3 treatment groups taken together, it had a significantly higher concentration for PHOS but not for BUN or CREA.

Discussion

The various therapeutic agents were selected based upon previous evidence of efficacy for T-2 toxicosis, as was the case of DEX and SAC, or due to a specific therapeutic goal, as was the case with fluid therapy (maintenance of AOM and cardiac output), BICARB therapy (maintenance of pHa), and METO (prevent the emesis associated with acute T-2 toxicosis). The MS was added to the slurry of SAC to serve as an osmotic cathartic and thereby decrease the intestinal transit time of the charcoal.

There was a overall significant treatment or time effect for many of the measured parameters. Most of the trends over time agree with previously reported values for swine given similar doses of T-2 toxin (Lorenzana et al., 1985a,b; Lundeen et al., 1986). Only those parameters that appeared to be affected by therapeutic intervention in this study, or those for which future studies are warranted, are discussed below.

Glucocorticosteroids are efficacious for the treatment of other circulatory shock states (Shatney, 1983; Schumer, 1983; Lefer and Spath, 1984). Efficacy is primarily attributed to stabilization of cell membranes, particularly lysosomal membranes (Lefer and Spath, 1984). Maintenance of lysosomal membrane integrity prevents the release of lysosomal hydrolases and the formation of cardioinhibitory factors such as myocardial depressant factor (MDF) (Lefer and Barenholz, 1972). The role that MDF plays in the pathophysiology of acute T-2 toxicosis is not clear, although a severe decline in pancreatic blood flow, noted to occur in swine given toxic doses of T-2 toxin by Lundeen et al. (1986), would likely predispose to its formation. If myocardial depressant factor plays a role in the pathophysiology of acute T-2 toxicosis, it would adversely affect cardiac performance. Therefore, glucocorticosteroids would be expected to preserve

cardiac function indirectly via cell membrane stabilization. In the present study, there was no significant treatment effect on indices of cardiac function such as CI, LVW, RVW, or SV between the positive control group and the other treatment groups given DEX as part of the therapeutic protocol. There was a significantly higher HR in the group given all therapy minus SAC + MS vs the remaining treatment groups taken together, although there was no difference detected between the positive control group and the same 3 treatment groups. The reason for the higher HR when SAC + MS was witheld from the treatment protocol is not clear, although the pigs in this group had a much lower mean survival time compared to the other treatment groups (Table I.21). However, this does not explain why there was no difference in HR between the positive control group and the 3 treatment groups, since the positive control group had the shortest mean survival time.

Fluid therapy is a major component of the treatment for circulatory shock (Kolata, 1980; Safar, 1982). The goal of fluid administration is to maintain an adequate intravascular volume, thus secondarily improving AOM and venous return to the heart. The need for fluid administration is not as controversial as the question of whether cystalloid or colloidal fluids are the most appropriate (Tranbaugh and Lewis, 1985; Dawson and Cowley, 1985). In the present study, intravascular volume expansion was attempted using normal saline. Other balanced cystalloid fluids such as lactated Ringer's were considered but not chosen due to the presence of lactate and potassium which may have aggravated lactic acidosis or hyperkalemia noted in swine previously dosed with T-2 toxin (Lorenzana et al., 1985a,b). The administration of large volumes of normal saline failed to significantly improve venous return to the heart as evidenced by a failure to reverse declines in AOM and CI. Based upon the large volume of fluid lost into the gastrointestinal tract due to the occurrence of severe, watery diarrhea and the presence of tissue edema upon post-mortem examination, it was concluded that substantial amounts of the administered fluid were lost from the intravascular space.

One hypothesized benefit from the administration of balanced crystalloid fluids in circulatory shock states is hemodilution (Safar, 1982). Decreased blood viscosity may be advantageous in the perfusion of areas with blood stasis and sludging and may improve tissue oxygenation. In the present study, the HCT (used as a measure of hemoconcentration) initially increased in all the experimental groups (Fig. I.35). This was likely due to catecholamine-mediated splenic contraction following toxin administration (Lorenzana et al., 1985a). By 2 to 3 hr, the HCT had begun to decline in each of the treatment groups, whereas it continued to increase gradually in the positive control group. There was a significantly lower HCT in the group given all therapy when compared to the positive control group. However, in the group given all therapy, the decrease in severity of hemoconcentration cannot be attributed solely to the administration of SAL since there was no significant difference in HCT between the group given all therapy minus SAL compared to the other 3 treatment groups taken together. This contrasts with the results for serum TP concentrations which can also serve as a measure of hemoconcen- tration. A significantly lower TP in the group given all therapy was noted as compared to the positive control group. In addition, there was a significant difference between the group given all therapy minus SAL and the remaining treatment groups in which SAL was included in the therapeutic protocol, indicating that the declines in TP were due to SAL administration. The modest decline in TP values for the positive control group and the group given all therapy minus SAL indicates that there may be some leakage of protein, especially albumin, from the intravascular space into the

extravascular space through damaged endothelial cells. This has been observed to occur in other shock states (Lefer, 1982). Given the failure of normal saline to maintain AOM, the use of colloidal solutions such as dextran, either alone or in combination with hypertonic saline, should be evaluated for the maintenance of MAP.

The cessation of urine production occurred in swine shortly after the administration of T-2 toxin iv (Lorenzana et al., 1985b). This was attributed to the decline in AOM, although an associated decrease in renal blood flow noted in swine given T-2 toxin iv (Lundeen et al., 1986) is also a likely contributing factor. In the present study, therapy was able to maintain urine production despite declines in AOM. The rate of urine production expressed as ml/min over the 8 hr observation period for Groups 1 to 5 was 0.45, 2.66, 3.21, 1.08, and 2.82, respectively. Thus, urine production for the treatment group not given SAL was less than one-half that of the other 3 treatment groups. However, urine production in the group not given SAL was still twice that of the positive control group. The amelioration of increases in serum K, PHOS, and CREA between the positive control group and the treatment groups is probably a reflection of increased urine production.

Acute T-2 toxicosis in swine and rats is characterized by lactic acidosis (Lorenzana et al., 1985a; Feuerstein et al., 1985). The replacement of base loss using BICARB is effective in counteracting the acidosis associated with other shock states (Kolata, 1980; Safar, 1982; Hardie and Rawlings, 1983). In the present study, aggressive BICARB therapy seemed to ameliorate the decline in pHa (Fig. I.34). In addition, lower LA production in the treatment groups, as compared to the positive control group, probably also contributed to a less severe decline in arterial pH. The observation of a significantly more severe decline in arterial pH in the group given all therapy minus BICARB as compared to the treatment groups given BICARB indicates the benefit from the administration of BICARB alone. Additional evidence for the beneficial effect of BICARB comes from the observation that increases in PaO_2 and declines in $PaCO_2$ were significantly less severe for the treatment groups given BICARB compared to the group given no BICARB. These blood-gas changes in the treatment groups given BICARB indicate less need for respiratory compensation in response to toxin-induced metabolic acidosis.

The efficacy of SAC for the treatment of acute T-2 toxicosis has been reported in experimental animals exposed to the toxin by the oral, sc, and iv routes (Galey et al., 1987; Fricke and Jorge, 1986; Poppenga et al., 1987a). In this study, as reported elsewhere, there was significantly prolonged survival in those groups in which SAC + MS was part of the treatment protocol vs the group which was given all therapy minus SAC + MS (Poppenga et al., 1987a). It is likely that the SAC was able to adsorb free and microbially deconjugated, epoxide-bearing metabolites of T-2 toxin within the intestinal tract and prevent their enterohepatic recirculation. This would be expected to lessen the local effects of free metabolites on the intestinal tract and, perhaps, systemic exposure to harmful metabolites, thus ameliorating pathophysiologic effects of the toxin. The only measured parameter which appeared to be adversely affected by the administration of SAC + MS was the serum Mg concentration. There was a slight decline in serum Mg concentration in the group given all therapy minus SAC + MS, whereas Mg concentrations were significantly elevated in the positive control and other treatment groups (Fig. I.36). Thus, the administration of MS caused an increase in serum Mg. The increase in serum MG in the positive control group may be a result of

cell destruction as hypothesized by Lorenzana et al. (1985b). The administration of other therapy, including SAC + MS, may minimize the serum Mg elevations due to cell damage although significant absorption of Mg from the intestinal tract may still occur. Elevations in serum Mg concentrations of the same magnitude as seen in this study have been associated with hypotension (Aikawa, 1981). The use of other osmotic cathartics such as sodium sulfate and sorbitol should therefore be considered instead of MS.

Observed changes in 2 parameters may warrant future therapeutic consideration. There was a significant decline over time in TOTCAL and GLUC concentrations irrespective of treatment given. Similar changes in TOTCAL have been observed in other circulatory shock states (Holcroft et al., 1980; Harrigan et al., 1983). The decrease in TOTCAL may be associated with an intracellular influx of calcium. It has been suggested that such a calcium influx may be a common final pathway leading to cell death (Shier, 1985). If this is the case, then attempts to block this intracellular movement should be considered. The use of slow calcium channel blockers has improved survival in other circulatory shock states (Hackel et al., 1981; Hess et al., 1983; Hess et al., 1985). However, the administration of the slow calcium channel blocker, dazemgrel, did not improve survival in rats given an otherwise lethal iv dose of T-2 toxin (Poppenga et al., 1987b). It is also interesting to note that amelioration of declines in arterial pH did not prevent or reverse declines in TOTCAL.

Decreases in plasma GLUC have been noted in endotoxic shock (Hinshaw, 1982). Declines are probably a reflection of both an increased need for energy and an impairment of gluconeogenesis (Filkins and Cornell, 1974; Hinshaw, 1982). It is likely that there is a cell energy deficit in acute T-2 toxicosis as has been proposed for other circulatory shock syndromes. Consideration should be given to assessing the efficacy of the provision of energy in treatment for acute T-2 toxicosis. However, the provision of high energy phosphate bonds in the form of ATP + $MgCl_2$ did not improve survival in rats given T-2 toxin (Poppenga et al., 1987b).

In summary, all the therapeutic combinations evaluated in this study had a beneficial effect on overall survival. The attempts to define the benefits in terms of amelioration of specific pathophysiologic events were not entirely successful perhaps due in part to the relatively small number of swine in each group and the variability among animals within groups in response to treatment. At the present time, the most appropriate treatment for acute T-2 toxicosis is nonspecific supportive care including glucocorticosteroids, fluids, BICARB, and the oral administration of intestinal adsorbents, such as SAC.

Acknowledgements

The authors are grateful to Dick Manuel and Renee Mariner for their excellent technical help. Our thanks also to Laura Beachy for typing the mauscript and table.

These studies were supported in part by the U.S. Army Medical Research and Development Command, Contract No. DAMD 17-82-C-2179 and 17-85-C-5224. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

Footnotes

[1]Yorkshire x Hampshire, Thrushwood Farms, Fairbury, IL.

[2]Rhusigen, Pitman-Moore, Inc., Washington Crossing, NJ.

[3]Thin layer and gas chromatography, Analytical Toxicology Laboratory, College of Veterinary Medicine, University of Illinois Diagnostic Laboratory, Urbana, IL.

[4]Perkin-Elmer Sigma 2000, Perkin-Elmer Corporation, Norwalk, CT.

[5]Fluothane, Fort Dodge Laboratories, Inc., Fort Dodge, IA.

[6]Compact 50, Foregger Co., Inc., Smithtown, NY.

[7]Ohio Anesthesia Ventilator, Ohio Medical Products, Madison, WI.

[8]Cardiatron Heart Monitor, Dyco, Inc., Scottsdale, AZ.

[9]A. Daigger and Co., Chicago, IL.

[10]Vetafil, S. Jackson, Inc., Bethesda, MD.

[11]Angiocaths, Deseret Medical, Inc., Sandy, UT.

[12]#12 French, Pharmaseal Corp., Toa Alta, Puerto Rico.

[13]Model #907 Infusion/Withdrawal Pump, Harvard Apparatus, Millis, MA.

[14]Gilson Medical Electronics, Inc., Middleton, WI.

[15]P23ID, Statham Medical Instruments, Gould, Inc., Oxnard, CA.

[16]Cardiogreen, Hynson, Westcott & Dunning, Inc., Baltimore, MD.

[17]DTL, Gilson Medical Electronics, Inc., Middleton, WI.

[18]Model DTCCO-07, Electronics for Medicine, Honeywell, New York, NY.

[19]IL813, Instrumentation Laboratory, Inc., Lexington, KY.

[20]IL237, Instrumentation Laboratory, Inc., Lexington, KY.

[21]Vacutainer, 6431 Red Top, Becton, Dickinson and Co., Rutherford, NJ.

[22]Hycel Super Seventeen Autoanalyzer, Houston, TX.

[23]Centriflo membrane cone, type CF23, Amicon Corp., Danvers, MA.

[24]305A Perkin-Elmer spectrophotometer, Norwalk, CT.

[25]Vacutainer, 6522 Grey Top, Becton, Dickinson and Co., Rutherford, NJ.

[26]Vacutainer, 6385 Lavender Top, Becton, Dickinson and Co., Rutherford, NJ.

[27]Cyanmethemoglobin method, Sigma Chemical Co., St. Louis, MO.

[28]Model ZBI, Coulter Electronics, Hialeah, FL.

[29]Sigma Chemical Co., St. Louis, MO.

[30]Statistical Package for the Social Sciences (SPSSX), SPSS Inc., Chicago, Illinoi .

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Table I.21 Swine therapeutic study: experimental groups and survival data

Group (n = 3)*	Treatment	Mean Weight (kg)	Survival Time (Hours)	Mean Survival Time (Hours)
1	Control T-2 + Metoclopramide	51.0	9.4, 7.8, 8.6	8.6
2	Metoclopramide Dexamethasone Normal saline and $NaHCO_3$ Activated charcoal + $MgSO_4$ (All therapy)	53.3	9.5, 48.0, 48.0	+
3	Metoclopramide Dexamethasone Normal saline and $NaHCO_3$ (No activated charcoal or $MgSO_4$)	45.5	15.8, 20.1, 18.0	18.0
4	Metoclopramide Dexamethasone Activated charcoal + $MgSO_4$ Normal saline (No $NaHCO_3$)	47.8	11.3, 22.5, 30.0, 48.0	+
5	Metoclopramide Dexamethasone Activated charocal + $MgSO_4$ $NaHCO_3$ (No saline)	53.6	23.5, 48.0, 48.0	+

*Treatment group 4: n = 4.
+No group means were calculated due to presence of censored observations.

Table I.22 Swine therapeutic study: drug administration protocol

Drug	Source	Dosage Regimen
Metoclopramide	Injectable form (5 mg/ml) courtesy of A. H. Robins Company.	1 mg/kg body weight iv immediately prior to T-2 toxin administration and 1/4 and 1-1/4 hr post-T-2 toxin.
Activated Charcoal	SuperChar® courtesy of Gulf BioSystems, Inc.	2 grams activated charcoal (dry weight) per kg body weight in 420 ml tap water po 1/2 hr and 4 hr post-T-2.
Magnesium Sulfate	Epsom salt, magnesium sulfate USP, purchased from Dow Chemical Company.	1/2 gram/kg body weight po mixed with activated charcoal slurry and administered 1/2 hr and 4 hr post-T-2.
Dexamethasone Sodium Phosphate	Azium S/P® (4 mg dexamethasone sodium phosphate/ml), purchased from Schering Corporation.	6 mg/kg body weight iv immediately and 4 hr post-T-2, then 4 mg/kg 8 and 12 hr post-T-2, followed by 2 mg/kg 16 and 20 hr post-T-2 and 1 mg/kg 24 hr post-T-2.
Sodium Bicarbonate	5 percent sodium bicarbonate injection, USP. Purchased from Abbott Laboratories.	Variable speed iv drip based on hourly arterial blood pH measurements. Started if pH < 7.350 and stopped if pH > 7.350.
Normal Saline	0.9 percent sodium chloride injection, USP. Purchased from Abbott Laboratories.	Rapid iv drip (gravity flow) as MAP* begins decline. Administration slowed to maintenance levels if MAP does not respond or if CVP + > 10 mmHg.

*Mean arterial blood pressure.
+Central venous pressure.

Figure I.31 Changes in cardiac index over time. No significant differences were detected among the various groups across all time points.

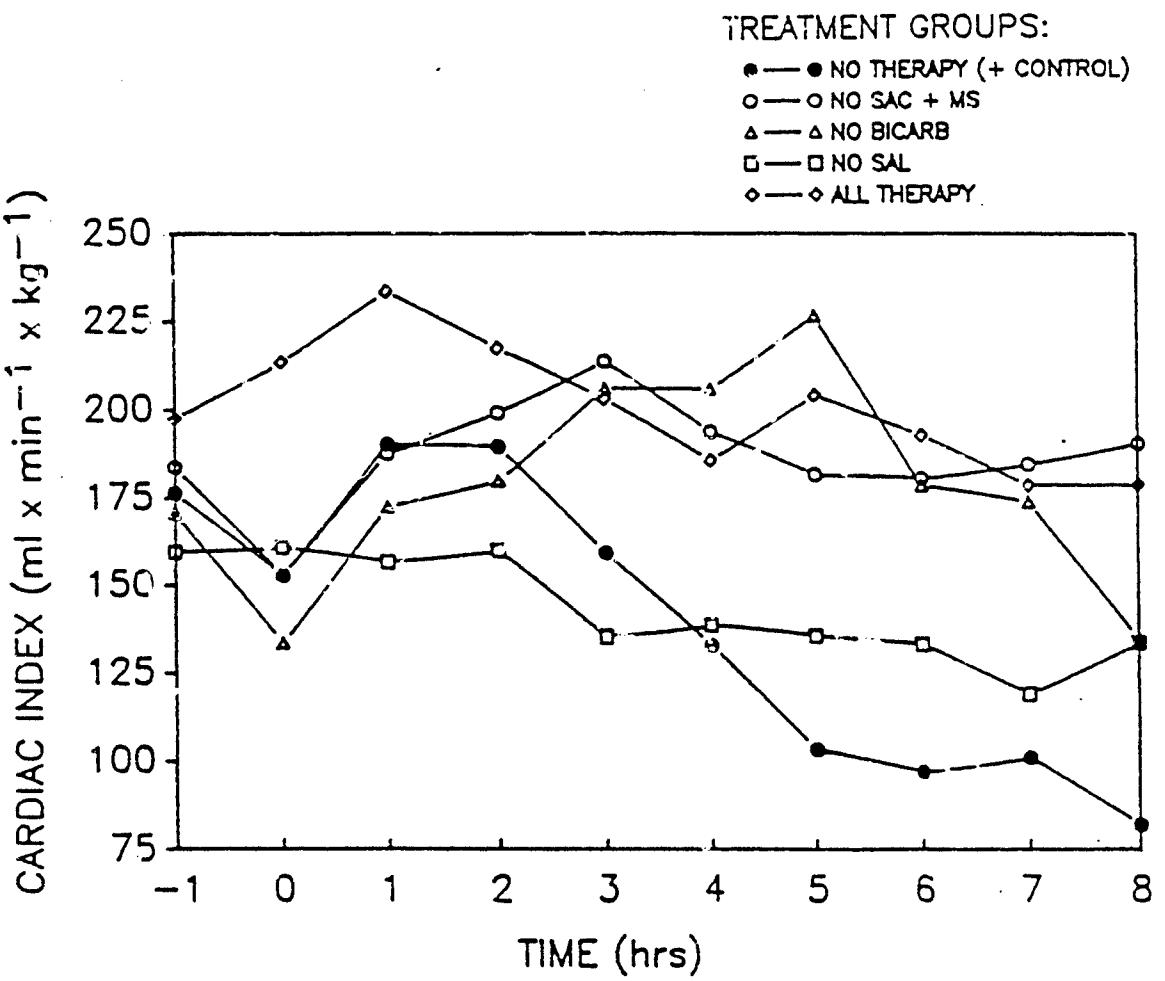


Figure I.32 Changes in aortic mean pressure over time. No significant differences were detected among the various groups across all time points. Across all treatments, there was a significant difference among time points ($p = 0.0001$).

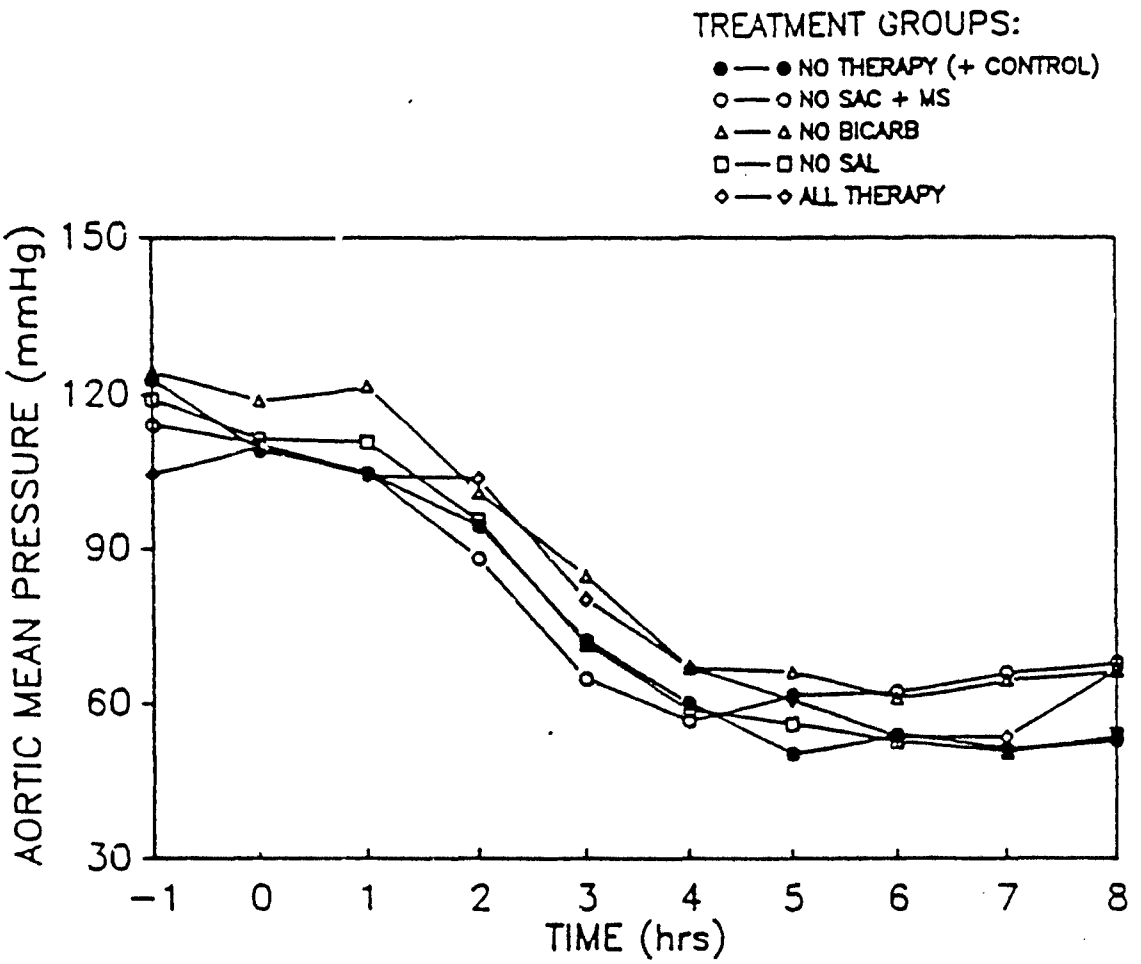


Figure I.33 Changes in arterial pH over time. There was a significant difference between the positive control group (no therapy) and the group given all therapy ($p = 0.071$). In addition, there was a significant difference between the group given all therapy minus BICARB and the other 3 treatment groups taken together ($p = 0.026$).

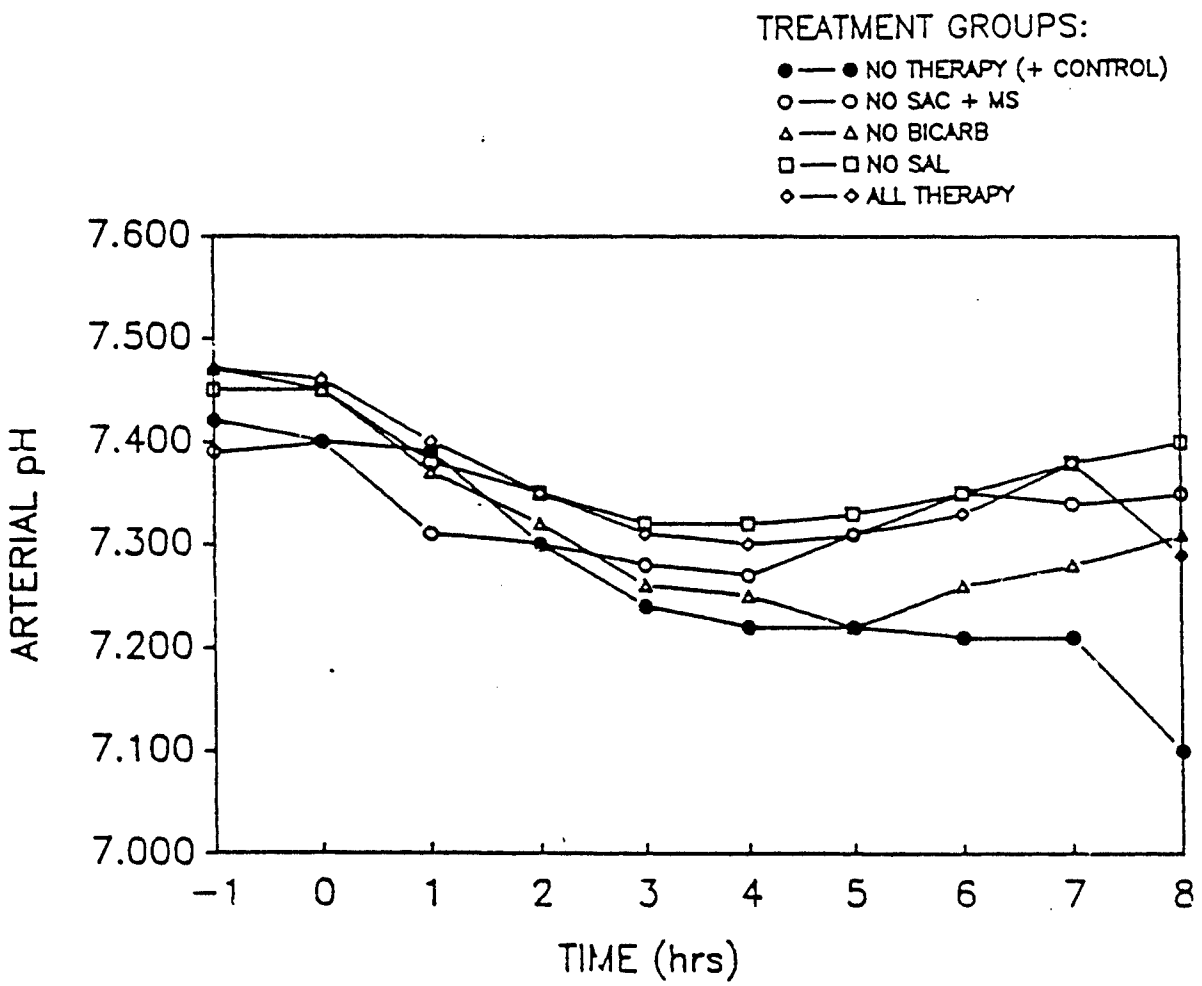


Figure I.34 Changes in plasma lactic acid concentrations over time. There was a significant difference between the positive control group (no therapy) and the group given all therapy ($p = 0.006$).

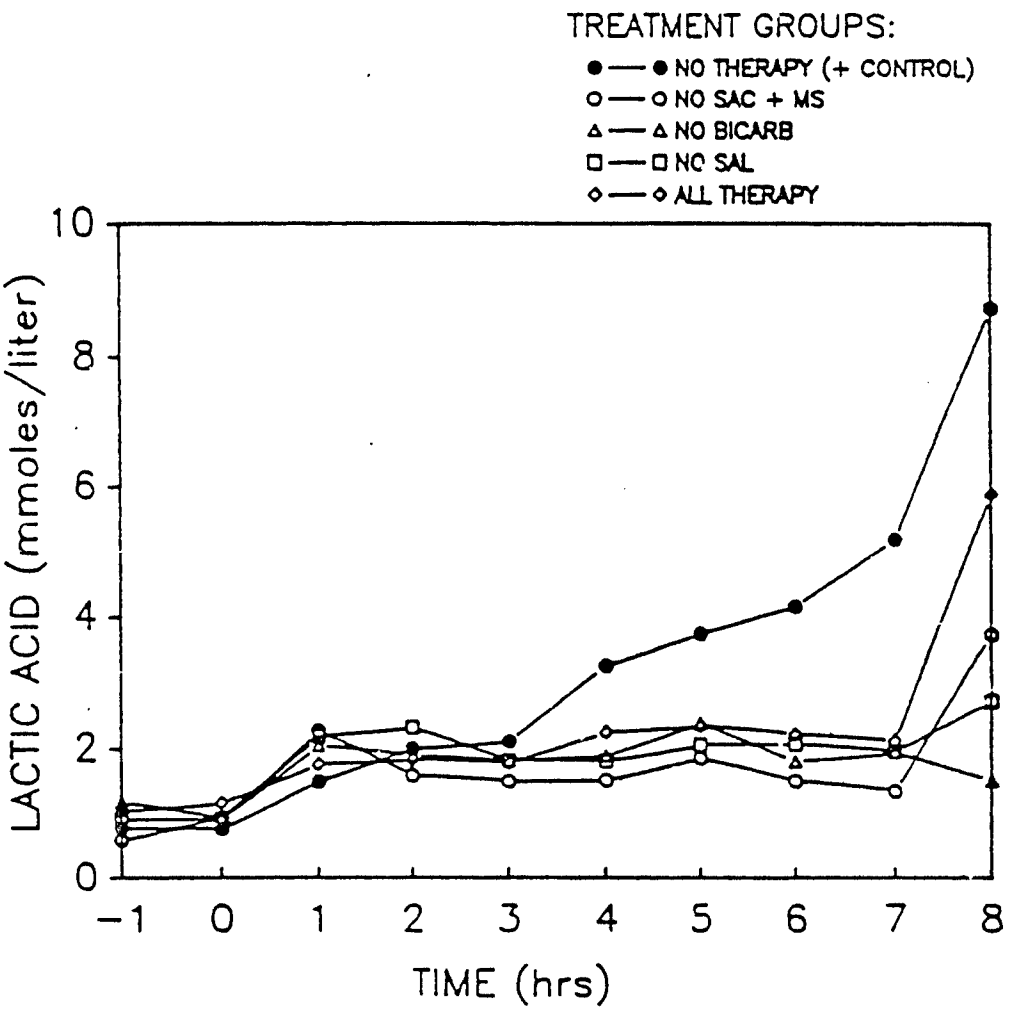


Figure I.35 Changes in hematocrit over time. There was a significant difference between the positive control group (no therapy) and the group given all therapy (p = 0.016). There was no significant difference detected between the group given all therapy minus SAL and the other 3 treatment groups taken together.

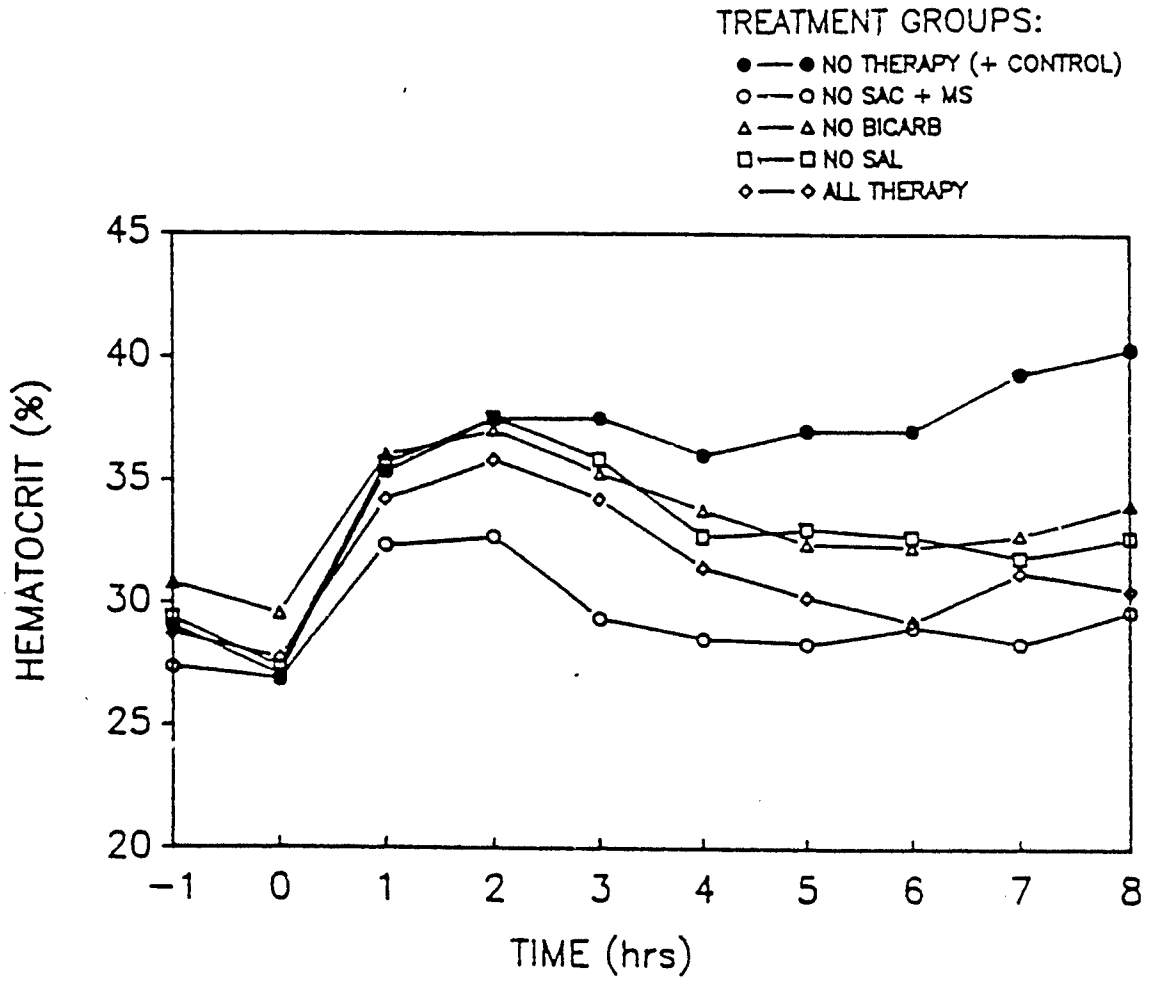


Figure I.36 Changes in serum magnesium concentrations over time. There was a significant difference between the positive control group (no therapy) and the group given all therapy minus SAC and MS ($p = 0.047$). In addition, there was a significant difference between the group given all therapy minus SAC + MS and the other 3 treatment groups taken together ($p = 0.008$).

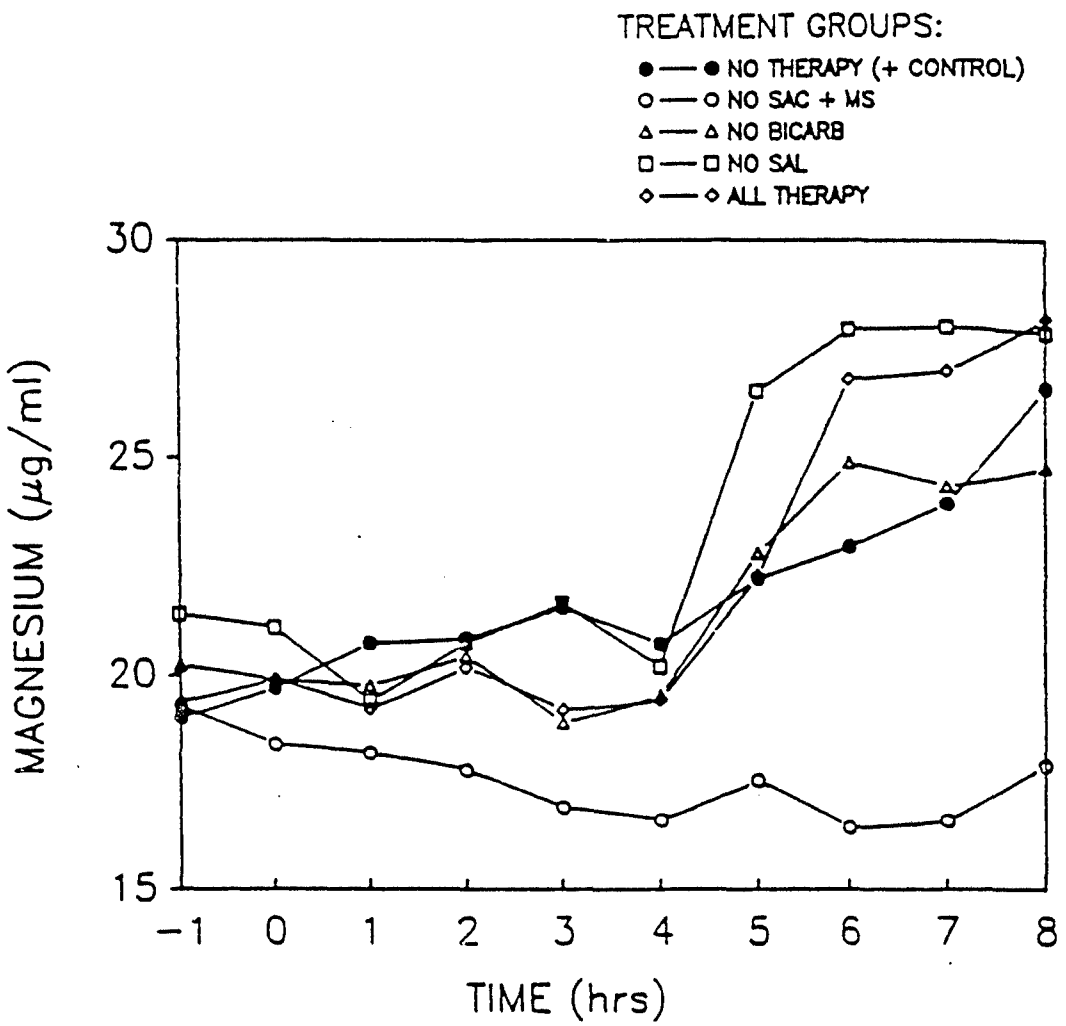


Figure I.37 Changes in total serum protein concentrations over time. There was a significant difference between the positive control group (no therapy) and the group given all therapy ($p = 0.002$). In addition, there was a significant difference between the group given all therapy minus SAL and the other 3 treatment groups taken together ($p = 0.003$).

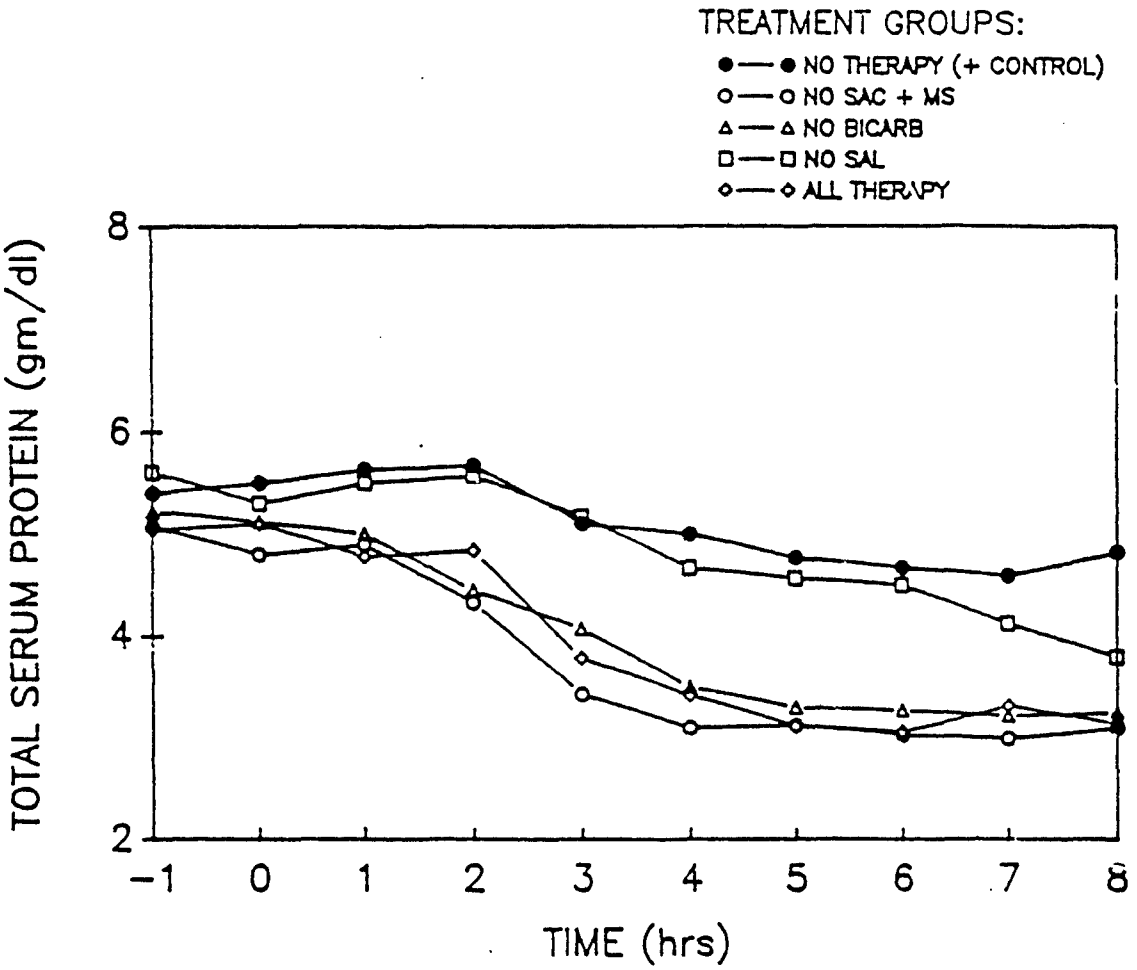


Figure I.38 Changes in serum calcium concentrations over time. No significant differences were detected among the various groups across all time points.

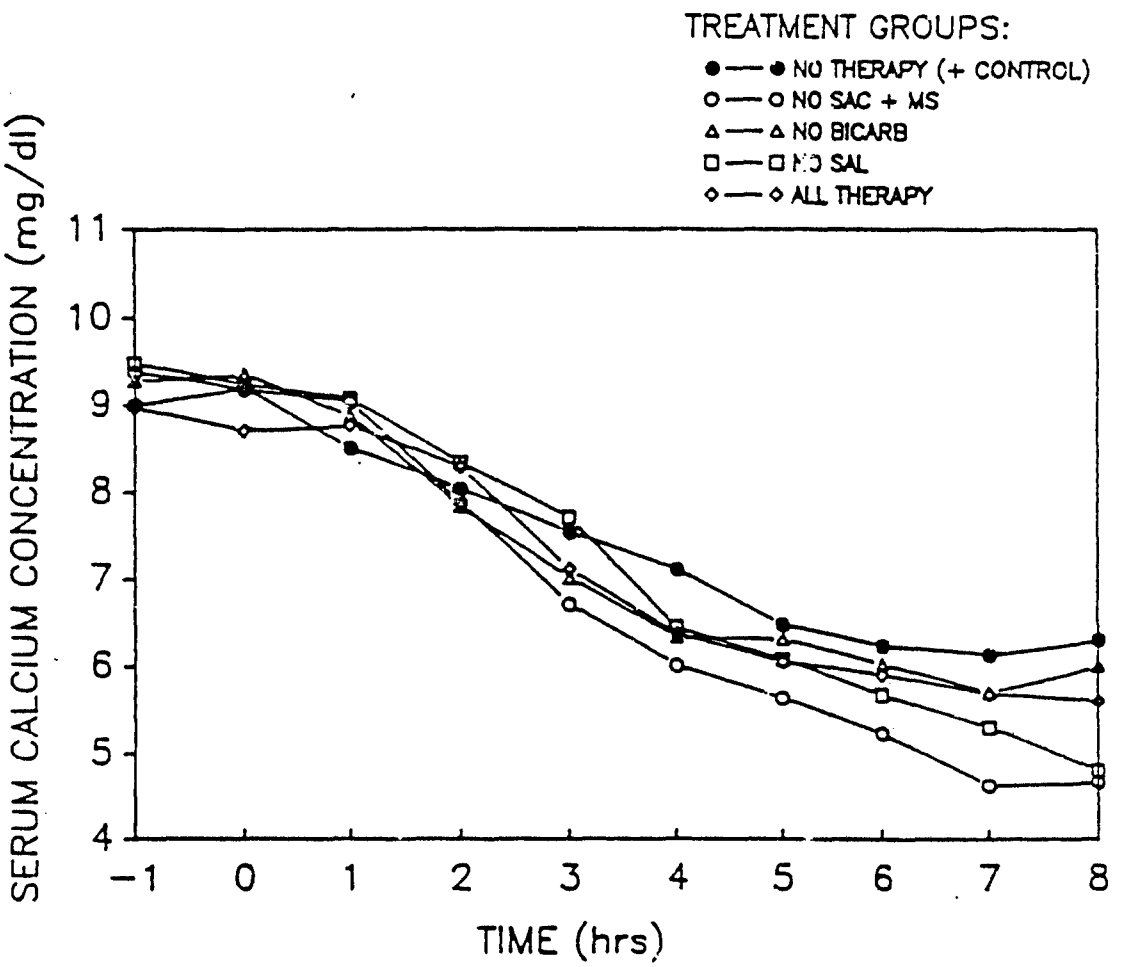
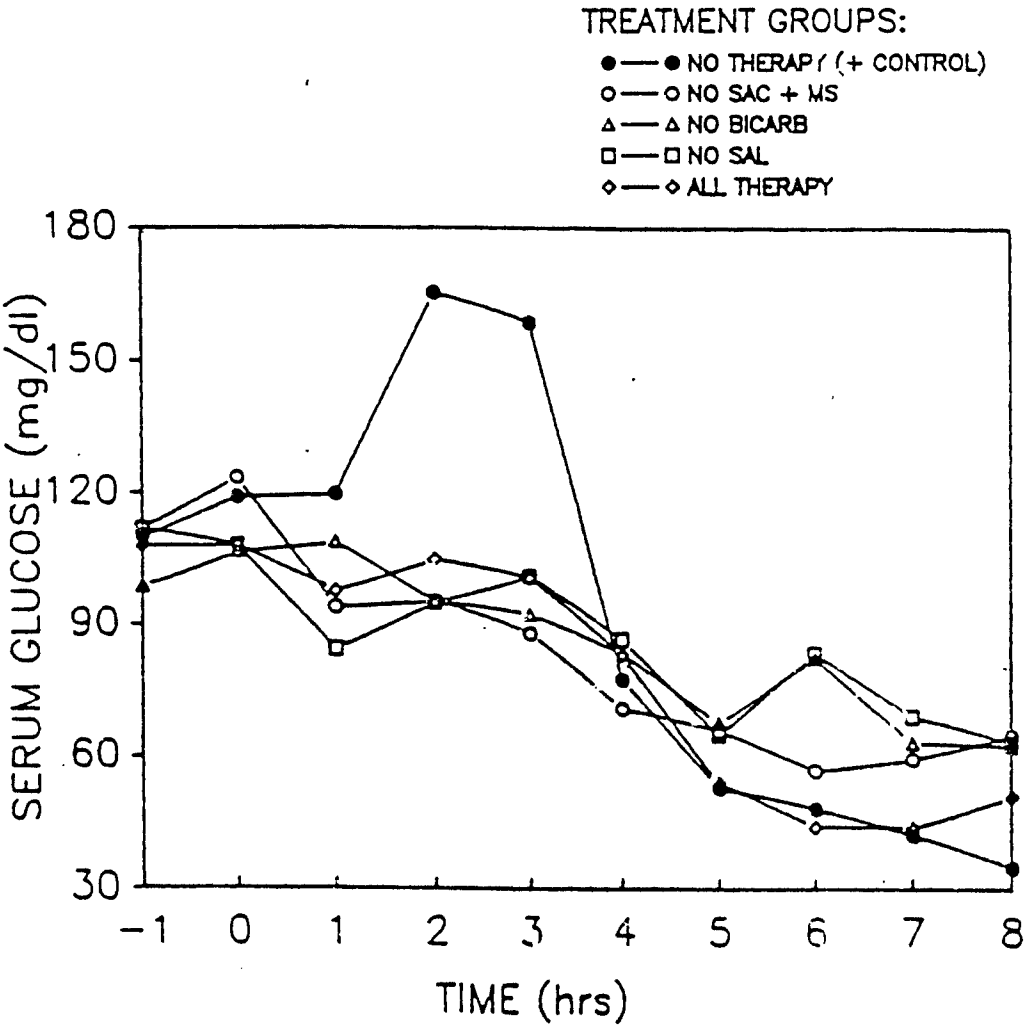


Figure I.39 Changes in plasma glucose concentrations over time. No significant differences were detected among the various groups across all time points.



J. Comparison of the Oral Adsorbents Superactivated Charcoal and Rohm and Haas Resin XE-348F

1. In vitro binding capacity for T-2 toxin.

by
Francis D. Galey, Steve Swanson, and Richard Lambert

The first in vitro experiment was designed to test the ability of ground Ambersorb XE-348F resin (Rohm and Haas, Philadelphia, PA) to remove T-2 toxin from solution. This was done as a preliminary step prior to comparing the ability of the resin and superactivated charcoal to bind T-2 toxin.

The binding ability of the resin for the toxin was measured by counting the amount of unbound tritiated T-2 toxin (^{3}HT-2) remaining in the supernatant of the resin-T-2 toxin solution. Six groups of 3 test tubes each were utilized. The first group contained 0.03 mg ^{3}HT-2 in 50 µl ethanol, 4 ml water, and no resin. It was used as a positive control standard against which the next 4 test samples could be compared. Each contained the same ingredients as the first group with the addition of 400 mg resin and various amounts of unlabeled T-2 toxin. The amount of unlabeled toxin added to each tube varied with the group while the volume was held constant. The sixth group of tubes was used for background counts, and each received only ethanol and deionized water.

The tubes were all gently mixed with an automatic rocker for 15 minutes and then centrifuged at 1,500 rpm for 5 minutes. The supernatant was filtered through 0.45 µm HPLC filters to remove remaining resin (and any bound T-2 toxin). Four hundred µl of the filtrate was placed in a 7 ml plastic counting vial to which 5 ml Scinti Verse™ liquid scintillation cocktail (Fisher Scientific Co., Fair Lawn, NJ) was added. The tubes were counted 24 hours later (to avoid chemiluminescence) for 2 minutes each in a liquid scintillation counter.

Raw counts per minute were corrected by subtracting from each test group the mean background count of group 6. The percent bound T-2 was then calculated for each tube as follows:

% Bound = [1 - (counts per minute test/mean counts per minute positive control)] x 100.

The mean and standard deviations for % bound were calculated. The results are presented in the following table:

Table I.23 *In vitro* binding of T-2 toxin by Ambersorb XE-348F

Group (n = 3)	Total mg T-2 Toxin	Ambersorb XE-348F	Ratio Resin:T-2	% T-2 Bound x ± (SD)
1. Control, no resin	0.03	0	NA*	NA*
2. Test	0.03	400 mg	13,333:1	99.87 (.04)
3. Test	0.30	400 mg	1,333:1	99.87 (.07)
4. Test	3.00	400 mg	1,333:1	99.73 (.04)
5. Test	30.00	400 mg	13:1	99.78 (.12)
6. Background control	0.00	0	NA	NA

*NA = not applicable

The Rohm and Haas resin removed greater than 99.5% of the T-2 in 15 minutes for all of the T-2 toxin groups tested down to a ratio of 13 parts resin to 1 part T-2 toxin. Therefore, it appears to be an effective adsorbent for T-2 toxin within the range of the resin:toxin ratios tested.

2. Comparison of the two adsorbents, SuperChar activated charcoal and Rohm and Haas Ambersorb XE-348F resin, for capacity to bind T-2 toxin

Procedure

A 4 x 4 design was utilized to compare the 2 adsorbents, SuperChar activated charcoal and Rohm and Haas Ambersorb XE-348F resin for their capacity to bind T-2 toxin *in vitro*. Four different quantities (50, 100, 200, and 400 mg) of adsorbent (wet weight) were suspended in 10 ml of water containing a constant amount of tritium labeled T-2 toxin plus unlabeled toxin sufficient to give 0.03, 0.3, 3.0, or 30 mg total T-2 toxin. Each treatment was run in triplicate.

After incubation at room temperature for 15 minutes, the mixtures were filtered through a 0.45 μ HPLC filter to remove suspended adsorbent. Aliquots of the filtrate were then removed and diluted with scintillation cocktail prior to counting for radioactivity on a liquid scintillation counter.

Results

At all 4 adsorbent concentrations (5, 10, 20, and 40 mg/ml), both SuperChar and Ambersorb XE-348F effectively removed > 99% of the T-2 toxin at the 3 lowest toxin concentrations (0.003, 0.03, and 0.30 mg/ml). Only at the highest toxin concentration of 3.0 mg/ml were differences in adsorption observed. SuperChar gave inconsistent results

at this toxin concentration, although the mean percentage of toxin removed exceeded 86% for all 4 SuperChar levels. With the Rohm and Haas resin, 77, 88, 98, and 99% of the toxin was removed at resin concentrations of 5, 10, 20, and 40 mg/ml, respectively.

Summary

Both SuperChar and Ambersorb XE-348F removed greater than 99% of the added T-2 toxin within 15 minutes at adsorbent to toxin ratios of from 13,333 to 16.7 to 1. At the highest concentration of T-2 toxin (3.0 mg/ml), the Rohm and Haas resin removed 99, 98, 88, and 77% of the toxin, which corresponds to resin:toxin ratios of 13.3, 6.67, 3.33, and 1.67, respectively.

3. In vitro adsorption of T-2 toxin by Rohm and Haas resin XE-348F and SuperChar superactivated charcoal

by
Cathy Knupp

This in vitro experiment was designed to compare the adsorptive capacities of ground Ambersorb XE-348F resin and SuperChar superactivated charcoal for T-2 toxin at 2 different pH conditions. The ability of the adsorbent to bind the toxin was measured by counting the amount of unbound tritiated T-2 toxin remaining in solution following filtration of the adsorbent-T-2 mixture. Table I.24 lists the ratios of adsorbent to toxicant evaluated, as well as the amounts of T-2 toxin and either SuperChar or resin.

The resin or charcoal suspension was prepared in distilled water for the pH 7 experiment and in 0.2 M acetic acid (pH 2.1, adjusted with 1.0 N HCl) in order to simulate pH conditions present in the stomach. T-2 toxin was dissolved in 20% ethanol and 1.0 ml placed in a disposable 17 ml test tube. A constant amount of tritiated T-2 (50 µl) was added to each sample and the solution vortexed. One ml of the adsorbent was added and vortexed, then the tubes were gently mixed for 15 minutes on a rocker mixer (final volume 2.0 ml, containing 10% ethanol). Each incubation was carried out in triplicate. Blank incubation tubes containing a low level of T-2 toxin and adsorbent but no radiolabeled material were used to determine background counts per minute (cpm). A set of 6 tubes containing a low concentration of T-2 toxin and radiolabeled T-2, but no adsorbent, were used as standards. At the end of the incubation period, the suspension was filtered through a 0.45 µ HPLC filter and 400 µl transferred to a glass scintillation vial. Liquid scintillation cocktail (4.5 ml, Scinti Verse™, Fisher Scientific Co.) was added and the samples counted for 1.0 minute. Table I.25 presents the mean percent and standard deviation of T-2 toxin adsorbed at various adsorbent:toxin ratios at either pH 7.0 or 2.1.

Table I.24 Adsorbent:T-2 toxin ratios, concentrations, and total quantities of adsorbent and toxin used.

Ratio of Adsorbent:T-2 (w/w)	Adsorbent		T-2 Toxin	
	mg/ml	Total mg	mg/ml	Total mg
2.5:1	5	10	2.0	4.0
5.0:1	5	10	1.0	2.0
10.0:1	5	10	0.5	1.0
20.0:1	5	10	0.25	0.5
40.0:1	5	10	0.125	0.25

Table I.25 Percent T-2 bound by Rohm and Haas resin or SuperChar at various adsorbent:toxin ratios and at pH 2.1 and 7.0. Values represent the mean of 3 replicates plus standard deviations.

Ratio of Adsorbent:T-2	Percent T-2 Toxin Bound			
	pH 2.1		pH 7.0	
	R & H Resin	SuperChar	R & H Resin	SuperChar
2.5:1	17.04 (0.83)	97.79 (0.59)	19.02 (1.99)	96.51 (1.01)
5.0:1	43.86 (2.28)	99.63 (0.07)	41.42 (2.19)	99.56 (0.32)
10.0:1	83.36 (0.91)	99.82 (0.04)	83.60 (1.75)	99.88 (0.05)
20.0:1	99.81 (0.05)	99.93 (0.03)	99.91 (0.03)	99.94 (0.03)
40.0:1	99.95 (0.02)	99.92 (0.04)	99.93 (0.04)	99.98 (0.02)

Summary

Both SuperChar and Ambersorb XE-348F removed greater than 99% of the added T-2 toxin within the 15-minute *in vitro* incubation period at adsorbent: toxicant ratios of 20:1 or greater. At lower ratios, however, SuperChar was the superior adsorbent, removing an average of 97% of the T-2 at a ratio of 2.5:1, compared to an average of only 17% bound by the resin at the same ratio. The percent bound data for SuperChar is in agreement with previous experiments. Rohm and Haas resin did not bind the T-2 efficiently at ratios less than 20:1. The increased amount of ethanol used to ensure complete dissolution of the toxin (10% final concentration in this experiment, versus 1% in previous studies) may have competed for adsorptive sites on the resin or caused the release of the toxin following initial binding. Simulation of acidic pH conditions present in the stomach did not significantly affect the binding of T-2 toxin by either SuperChar or Ambersorb XE-348F.

The effect of ethanol concentration on the binding of T-2 toxin by SuperChar and Ambersorb XE-348F was investigated following the initial adsorption study. Ethanol concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0% in the final incubation mixture were compared. An adsorbent:toxin ratio of 5:1 was used. Incubations were carried out in triplicate according to the same protocol as described earlier. Table I.26 presents the mean percent and standard deviation of T-2 toxin bound by either SuperChar or Ambersorb XE-348F. Incomplete dissolution of T-2

toxin in previous studies probably resulted in binding values by the Rohm and Haas resin that were artificially high.

Table I.26 Percent T-2 bound by Rohm and Haas resin or SuperChar at various ethanol concentrations using an adsorbent:toxin ratio of 5:1. Values represent the mean of 3 replicates plus standard deviations.

Percent of Ethanol	Percent T-2 Bound	
	Rohm and Haas	SuperChar
0.5	39.35 (3.00)	99.92 (0.01)
1.0	43.56 (1.89)	98.56 (0.90)
2.5	46.11 (3.51)	99.45 (0.40)
5.0	47.48 (2.25)	99.71 (0.22)
10.0	46.37 (2.55)	99.64 (0.07)
20.0	37.64 (1.69)	97.89 (0.30)

4. In vivo treatment efficacy in rats for oral exposure to T-2 toxin

by
Richard Lambert and Barbara Kindler

Introduction

Rohm and Haas resin XE-348F is a polymer carbon adsorbent which is regarded as an alternative to granular-activated carbon adsorbents.

The objectives of the initial in vivo studies were to: 1) determine if oral Ambersorb resin XE-348F therapy was of any benefit in prolonging the survival of rats orally exposed to an LD_{99} dose of T-2 toxin, and 2) employ a range of doses of Ambersorb resin in an attempt to define a dose that would be effective in preventing deaths in 50% of the T-2 toxin-exposed animals (ED_{50}).

Materials and Methods

Experiment 1

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 200 to 250 g were given free access to food (Wayne Lab Blox) and water for 1 week prior to experimentation. Food was withdrawn at 12 to 14 hours before dosing. The animals were given 8 mg/kg body weight T-2 toxin (20% ethanol:water) orally via gavage needle followed by ground Ambersorb XE-348F (10% in water) at doses indicated in the tables. All animals within an experiment received the same total volume of treatment solution on a ml/g body weight basis and were observed for deaths for 7 days. Animals which died were necropsied in order to establish that the esophagus had not been perforated and the toxin and resin were delivered to the stomach. Data from improperly dosed animals were not included in the analyses.

Experiment 2

This study was essentially a repeat of Experiment 1 except that: 1) female rats were used; 2) there were no signs of SDA virus infection; and 3) the doses of Ambersorb were shifted slightly, i.e., because of the results from Experiment 1, the high dose was dropped in favor of a lower dose.

Experiment 3

In this experiment we explored the practicality of using higher doses of the resin within the limitations of a 1-time maximum toxin and treatment volume that could be given to the rats. Female rats were given T-2 toxin as described in Experiment 1 and treated as indicated in Table I.27.

Experiment 4

This represented a preliminary attempt to compare the relative efficacy of SuperChar superactivated charcoal (SCSAC) and Ambersorb resin XE-348F (Resin) in prolonging the survival of rats dosed orally with T-2 toxin. Female rats were given 8 mg/kg T-2 toxin orally followed by 1 of the treatments as indicated in Table I.28.

Data from all survival studies were analyzed using Gehan's Generalized Wilcoxin Test (Knapp and Wise, 1985) which takes into account censored data (survival for the duration of the experiment). Results were considered significant at $P < 0.05$ for all comparisons with control times.

Results. The results of Experiments 1 through 4 are given in Tables I.27 through I.30.

Table I.27 Dose groups, survival times and rates from Experiment 1.

Dose of Ambersorb	0.0 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg	2.0 g/kg
	Survival Time (hours)				
	23.7	161	161	161	161
	18.8	161	161	161	161
	14.3	161	161	161	161
	21.8	161	161	161	161
	22.8	161	161	161	161
	12.5	84.3	161	161	161
	16.5	161	12	16.5	38.5
	120	161	161	161	161
	18.8	161			
	13.3				
Ratio: Surviving/Dosed	0/10	8/9*	7/8*	7/8*	7/8*

*Survival times significantly different from the T-2 positive control group

The day after the experiment, some of the rats had signs consistent with infection by sialodacryoadenitis (SDA) virus. All resin treatment groups had significantly prolonged survival times in comparison to the T-2 dosed positive control rats. As a result of the consistently high survival rates, it was not possible to calculate an ED_{50} dose of Ambersorb. The role that the viral infection may have played in these results in unknown.

Table I.28 Dose groups, survival times and rates from Experiment 2.

Dose of Ambersorb	0.0 g/kg	0.10 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg
	Survival Time (hours)				
	14.3	14.3	13.8	11.3	17.3
	14.3	13.3	11.3	15.5	16.3
	14.3	13.3	14.3	17.0	11.3
	14.5	20.0	15.8	20.5	22.3
	16.3	18.8	21.3	11.3	28.3
	13.5	13.3	11.3	11.3	35.5
	14.3	11.3	14.5	14.3	44.8
	15.8	16.3	26.8	168	168
	14.5	11.8	29.0		168
	15.8	16.8			168
Ratio: Surviving/Dosed	0/10	0/10	0/9	1/8	3/10

Survival rates were much poorer than Experiment 1. Statistically, the 1.0 g/kg dose of Ambersorb was the only one which significantly prolonged survival. Because survival was so poor, it was not possible to calculate an ED_{50}.

Table I.29 Dose groups, survival times and rates from Experiment 3.

Dose of Ambersorb	1.0 g/kg	2.0 g/kg	3.0 g/kg
	Survival Time		
	40.8	19.3	25.5
	74	27.0	57.75
	75	20.3	21.0
	75	34.5	21.0
	75	75	75.0
	75		75
Ratio: Surviving/Dosed	5/6	1/5	2/6

Necropsy of all animals suggested that the concentrations of resin (20%) used in the 2 highest doses resulted in gastric retention and poor gut passage of the material. The 1.0 g/kg dose (10% resin in water) was highly efficacious.

We subsequently dosed rats with the higher concentrations of resin (2 and 3 g/kg) but no T-2 toxin and killed them sequentially over 8 hours. The resin was not retained in the stomach and appeared to be preceding normally through the gastrointestinal tract. Hence, the poor passage of the material at the higher concentration in Experiment 3 was probably related to T-2 toxin exposure.

Table I.30 Dose groups, survival times and rates from Experiment 4.

Treatment	T-2 Toxin Positive Control T-2 Toxin	1 g/kg 10% solution SCSAC	1 g/kg 10% solution Resin	1 g/kg 20% solution Resin
	Survival Time (hours)			
	11.6	132	132	132
	11.6	132	132	24.4
	11.7	132	26.5	132
	11.4	132	132	23.1
	11.5		132	132
	10.6			42.5
	10.7			132
Ratio: Surviving/Dosed	0/7	4/4*	4/5*	4/7*

*Survival times significantly longer than T-2 dosed positive control rats.

Each treatment resulted in statistically significant prolonged survival times relative to the positive control group, but the treatments were not different from each other.

Experiment 5

These studies were designed to assess the repeatability of information obtained from previous studies, i.e., to: 1) determine if oral Ambersorb resin XE-348F therapy was of any benefit in prolonging the survival of rats orally exposed to an LD_{99} dose of T-2 toxin, and 2) employ a range of doses of Ambersorb resin in an attempt to define a dose that would be effective in preventing deaths in 50% of the T-2 toxin-exposed animals (ED_{50}).

Experimental methods were similar to those described in Experiment 1, except that female rats were used.

Results.

Table I.31 Dose groups, survival times and rates from Experiment 5.

Dose of Ambersorb	0.0 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg
	Survival Time (hours)			
	10.8	11.9	19.0	10.3
	10.6	10.8	11.7	10.5
	10.3	11.8	10.2	10.6
	10.7	11.1	25.4	10.4
	11.5	24.1	9.8	13.5
	10.4	23.3	20.0	11.2
	11.6	9.3	47.7	10.1
	10.1	11.0	161.0	25.0
	11.6	40.1	161.0	9.4
	9.4			161.0
Ratio: Surviving/Dosed	0/10	0/9	2/9	1/10
Percentage Survival	0%	0%	22%	10%

The results were consistent with the earlier resin study using virus-free female Sprague-Dawley rats (Experiment 2). The combined survival results of the 2 groups are illustrated in the following table.

Table I.32 Combined results of Experiments 2 and 5. Dose groups, survival times, and rates.

Dose of Ambersorb	0.0 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg
Ratio: Surviving/Dosed	0/20	0/18	3/17	4/20
Percentage Survival	0%	0%	17.7%	20%

Survival times were significantly greater only in the 0.25 g/kg and 0.5 g/kg groups (not the 1 g/kg group) when compared to the positive control group receiving no Ambersorb resin. Though survival times were significantly longer in those 2 groups, the survival rate was poor in all groups and much poorer than that obtained in previous work with comparable doses of superactivated charcoal (Galey et al., 1987).

Experiment 6

A second group of female Sprague-Dawley rats was used to assess whether survival times or rates could be improved by orally dosing with the Ambersorb resin immediately prior to oral dosing with T-2 toxin. The doses of resin and T-2 toxin were similar to those described in Experiment 1. The results are given in Table I.33.

Table I.33 Dose groups, survival times and rates from Experiment 6.

Dose of Ambersorb	0.0 g/kg	0.25 g/kg	0.5 g/kg	1.0 g/kg
	Survival Time (hours)			
	11.8	12.7	10.5	168.0
	10.4	31.4	168.0	168.0
	10.0	15.7	168.0	168.0
	10.0	11.7	168.0	168.0
	10.2	90.6	168.0	168.0
	11.7	168.0	168.0	168.0
	13.3	168.0	168.0	168.0
	10.2	168.0	168.0	168.0
	11.2	168.0	168.0	168.0
	10.4	168.0	168.0	168.0
			168.0	168.0
Ratio: Surviving/Dosed	0/10	5/10	10/11	11/11
Percentage Survival	0%	50%	91%	100%

The survival times for the T-2 positive control group were very consistent and similar to those obtained previously. However, when given prior to oral T-2 toxin, each dose of Ambersorb resin XE-348F significantly improved survival rates and times when compared to the positive control rats. Moreover, there was an improvement in survival with increasing doses of the resin.

Earlier work with virus-free rats had suggested that an oral dose of 1 g/kg of the ground resin significantly improved survival rates and times of rats given oral lethal doses of T-2 toxin (11 survivors out of 22 treated rats). Though survival was not as good as with superactivated charcoal, the results indicated that further study of the resin was warranted. Completion of studies using larger numbers of virus-free rats has resulted in consistently less favorable results except when the animals were pretreated with resin. Since the initial *in vitro* work had indicated that the resin and superactivated charcoal had comparable binding capacities for T-2 toxin, we wondered whether there was an interaction with fluids in the stomach that made the resin less effective. We believe that the *in vitro* work described earlier in this report, which indicates that superactivated charcoal has greater binding capacity for T-2 toxin, may help to explain the inconsistencies in the *in vivo* results.

It is also possible that the time allowed to elapse between administration of the toxin and the resin has a significant effect on the outcome, even if there is a delay of only a few minutes. We do not know how rapidly the resin binds the toxin. If the process is slow, significant amounts of toxin may be absorbed. That possibility, coupled with the relatively less efficient binding capacity relative to superactivated charcoal, may be responsible for the inconsistent efficacy of the resin.

Experiment 7

This was a direct comparison of treatment efficacy of Ambersorb and SuperChar, when given immediately after an oral lethal dose of T-2 toxin. Pertinent experimental parameters were as indicated in Experiment 1, except that female rats were used.

Results. Both the Ambersorb resin and SuperChar superactivated charcoal were effective in preventing deaths with no statistical difference between the 2 treatment groups (see Table I.34).

Table I.34 Results of Experiment 7, Comparison of Ambersorb and SuperChar.

	Treatment		
	Control	Ambersorb	SuperChar
Survived/Dosed	0/10	13/13	15/16
Percent Survival	0%	100%	94%

Experiment 8

This study was similar to Experiment 7 except that there were additional groups where the treatments were delayed until 1 and 3 hours after the toxin.

Treatments. 1) Ambersorb XE-348F resin (ground) given at a dose of 1 g/kg body weight as a 10% suspension via gavage needle immediately (0 hour), 1, and 3 hours after T-2 toxin; or 2) SuperChar superactivated charcoal given at a dose of 1 g/kg body weight as a 10% suspension via gavage immediately (0 hour), 1, and 3 hours after T-2 toxin.

Results.

Table I.35 Treatments, times of treatments, survival times and rates from Experiment 8.

T-2 Positive Control	Ambersorb			SuperChar		
	Treatment Time Post-T-2 Toxin Administration (hours)					
0	0	1	3	0	1	3
10	168	14	11	168	12	11
11	168	17	15	168	23	14
14	168	24	11	168	26	10
12	168	13	10	168	13	9
18	168	9	10	168	16	9
11	168	11	11		18	9
12		10	10		11	10
11		11	13		14	10
11		39	10		21	36
11		10	14		12	11
			12			12
			13			
Survived/ Died 0/10	6/6	0/10	0/12	5/5	0/10	0/11
Percent Survival 0%	100%	0%	0%	100%	0%	0%

When compared to the controls, only 1 Ambersorb-treated group had a significantly prolonged survival time, the one which received the resin immediately (within 1 to 2 minutes of the toxin).

The rats which received SuperChar superactivated charcoal immediately and 1 hour after the toxin had significantly prolonged survival times in comparison to the T-2 toxin-positive control group. Survival times for the group which received charcoal 3 hours after the toxin were not prolonged at a significance level of $P < 0.05$, but they approached that level ($P = 0.065$).

The results reported here differ somewhat with those of Galey et al. (1987), where it was shown that delaying superactivated charcoal therapy for as long as 3 hours resulted in a significant improvement in survival rates and times.

As indicated by most of our previous work, the Ambersorb resin was an effective therapy (as measured by survival times and rates) when given at 1 g/kg body weight as a 10% solution immediately after the toxin. Although the resin and superactivated charcoal were equally effective when given immediately, when given after a lapse of 1 or 3 hours, the charcoal was of significantly greater benefit. This may be a reflection of the slightly greater binding capacity of the charcoal, as shown in the in vitro experiments.

Experiment 9

The Ambersorb XE-348F resin and SuperChar superactivated charcoal were compared with respect to their efficacy in treating rats given an oral lethal dose of T-2 toxin. Female Sprague-Dawley rats ranging in weight from 200 to 275 g were given 8 mg/kg body weight T-2 toxin followed immediately by gavage with either 1.00, 0.75, 0.50, 0.25, or 0.10 g/kg of the resin or charcoal. All rats, including the positive controls, received the same volume of solution on a mg/kg basis and were monitored for at least 168 hours. The results are summarized in the following table.

Table I.36 Doses and associated survival rates from Experiment 9.

Treatment	Survivors/Dosed	Percent Survivors
Ambersorb XE-348F (g/kg)		
1.00	15/17	88
0.75	12/17	71
0.50	11/19	58
0.25	7/17	41
0.10	0/17	0
Superactivated Charcoal (g/kg)		
1.00	11/11	100
0.75	10/12	83
0.50	9/11	82
0.25	10/11	91
0.10	5/11	46
Positive Control (deionized water)	0/16	0

Calculating from the effective dose curves, the ED_{50} values and 95% fiducial limits were 0.40 g/kg (0.28, 0.52) for the resin and 0.09 g/kg (0.003, 0.18) for the superactivated charcoal.

The results, which were consistent with previous studies, indicate that a dose of 1.00 g/kg of the resin was fairly effective at protecting against an oral LD_{99} of T-2 toxin. At lower doses, it was proportionately less effective. Superactivated charcoal is much more effective than the resin, providing approximately 90% survival at doses as low as 0.25 g/kg body weight, and it has an ED_{50} value which is 4 times lower than the resin.

Summary

The combined data from the *in vitro* and *in vivo* work indicate that at high toxin to binding agent ratios, superactivated charcoal is more effective at adsorbing the toxin than the resin.

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K. Dexamethasone Therapy for T-2 Toxicosis in Rats

1. Pharmaacokinetics of intravenously and intramuscularly administered dexamethasone in rats

by
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Abbreviations:

ka, first-order absorption rate constant; β, elimination rate constant; B, intercept on y axis as related to beta; α, distribution rate constant; A, intercept on y axis as related to alpha; AUC, area under the plasma concentration-time curve; AUMC, area under the moment plasma concentration-time curve; Vd_{area}, apparent volume of distribution using area under the curve; Vd_{SS}, apparent volume of distribution at steady state; Cl, total clearance; AIC_1, Akaike's information criterion for one compartment model; AIC_2, Akaike's information criterion for two compartment model; F, absolute bioavailability; D_{10}/D_2, ratio of Dose 10 mg/kg/Dose 2 mg/kg; (T), trapezoidal method, a model independent technique for calculation of parameters; RP-HPLC, reverse phase high performance liquid chromatography; RIA, radioimmunoassay; $t_{1/2}$, elimination half-life; C_p, plasma concentration.

Abstract

The pharmacokinetics of dexamethasone in rats was investigated. The kinetic parameters following im and iv administration of dexamethasone at 10 mg/kg were statistically indistinguishable. The plasma half-life of dexamethasone was 2.6 h with a total body clearance of 0.266 l/kg/h and a volume of distribution of 1.1 l/kg after either iv or im administration. There was some suggestion of dose-dependent pharmacokinetics. The results obtained by reverse phase high performance liquid chromatography (RP-HPLC) were similar in magnitude and variability to published results obtained by radioimmunoassay (RIA). Allometric relationships were developed for clearance, CL (l/h) = 0.255 W using our results and published pharmacokinetic and body mass, W (kg), data for humans, cattle, dogs, and horses. These data show that the rat may be an acceptable model for the study of human therapeutic dosage regimens with dexamethasone because of the similarity in its pharmacokinetic parameters with those of humans.

Introduction

Dexamethasone alcohol (dexamethasone), one of several potent synthetic corticosteroids, is 25 to 30 times as potent as hydrocortisone on a dose equivalent basis (Haynes and Murad, 1985). Corticosteroids are widely used in medicine for their anti-inflammatory, antiallergenic, and antirheumatic properties. Dexametnasone has predominantly antiinflammatory (glucocorticoid) activity with little or no sodium retention (mineralocorticoid) activity (Haynes and Murad, 1985).

The pharmacokinetics of dexamethasone has been studied in humans (Hare et al., 1975; Tsuei et al., 1979,1980), cattle, dogs and horses (Toutain et al., 1982,1983,1984). Varma and Mulay (1980) studied the pharmacokinetics of dexamethasone administered iv to male Sprague-Dawley rats. Hare et

al. (1975) and Varma and Mulay (1980) used RIA methods for determining plasma dexamethasone concentrations while the other authors used RP-HPLC. Cross reactivity with endogenous steroids may occur during RIA determinations (Hichens and Hogans, 1974) and can impair analytical accuracy. Reverse phase HPLC is the recommended analysis method for dexamethasone in biological fluids because of its good specificity and high sensitivity (Cham et al., 1980; Cairns et al., 1983; Plezia and Berens, 1985).

This study was undertaken to: 1) compare the pharmacokinetic disposition of iv and im administered dexamethasone in rats, 2) examine for dose-dependent pharmacokinetics of dexamethasone in rats, 3) compare pharmacokinetic parameters obtained by the RP-HPLC method with published values obtained by RIA, and 4) develop allometric models for the pharmacokinetic parameters.

Methods

Dexamethasone sodium phosphate, 9-fluoro-11β,17-dehydroxy-16α-methyl-21-(phosphonooxy)pregna-1,4-diene-3,20-dione disodium salt (Decadron, 24 mg/ml), was obtained from Merck, Sharp, and Dohme (West Point, PA). Each 24 mg of dexamethasone sodium phosphate was equivalent to 19.98 mg of dexamethasone alcohol. Doses are reported as the dexamethasone alcohol.

Animals

Virus-free female Sprague-Dawley rats weighing 225 to 300 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were acclimatized to laboratory conditions (12 h light; 24°C) for 10 days. They were provided with Lab-blox, a standard commercial diet (Wayne, Chicago, IL), and tap water ad libitum.

Surgery

Rats were anesthetized with 40 to 50 mg/kg of pentobarbital (Abbott, Chicago, IL) given ip. Cannulas were surgically implanted in both the left common carotid artery and the left jugular vein (Weeks and Davis, 1964) 24 to 36 h before administration of dexamethasone. These cannulas were externalized between the shoulder blades and sutured in place, after which the animals were housed individually. Cannula patency was maintained by filling them with a heparinized saline solution (500 IU/100 ml 0.9% sodium chloride) which was removed twice daily, followed by flushing with 0.2 cc of 0.9% saline. During blood sampling periods, animals were restrained in rat holders for the first 40 min and then returned to their individual cages where food and water was available ad libitum. The remaining samples were collected from the animals with minimal restraint.

A predetermined dose (2 or 10 mg/kg) of dexamethasone was drawn into a 0.5 cc disposable syringe and weighed on an analytical balance. The syringe was reweighed after dosing to determine the actual quantity administered to each animal.

Prior to dosing with dexamethasone, the heparin solution in both cannulas was replaced with saline. Dexamethasone was administered into the jugular vein or im into the left hind leg. Serial blood samples (0.30 ml) were collected from the carotid artery into heparinized disposable

plastic syringes and transferred to microtest tubes. The blood sample volume was replaced with an equal volume of saline infused into the carotid artery. Blood samples were immediately chilled on ice and then centrifuged at 2900 rpm for 5 min at 5 to 10°C. Plasma samples were stored at -20°C for 1 to 60 days before analysis. At the end of the blood collection period, each animal was given a blood transfusion of freshly heparinized blood (obtained by cardiac puncture from euthanized donor rats) equal to the total volume of blood collected.

Pharmacokinetic Studies

Three studies were carried out. Study 1 compared the pharmacokinetics of im with iv administration. Studies 2 and 3 examined for dose-dependent pharmacokinetic parameters after iv and im administration, respectively. Rats were randomly assigned to one of three 2 x 2 Latin squares. Blood samples were collected at 0 (during surgery) and at 5, 20, and 40 min and 1, 2.5, 4, 6, 8, 10, and 14 h following dexamethasone administration. A washout period of 72 h was allowed between doses in the crossover studies.

Animals with a nonfunctional arterial cannula or which did not survive the initial crossover were replaced. The original crossover design was then repeated with both treatments administered to the replacement animal; a second subject was used as a control for treatment effect. Such replacement resulted in unequal numbers of animals between groups in a given experiment. In the first study, 1 group of rats (n = 4) received 10 mg/kg dexamethasone iv and a second group (n = 5) received 10 mg/kg im. Study 2 used one group of rats (n = 4) given 10 mg/kg dexamethasone iv and another group (n = 7) given 2 mg/kg iv. In the third study, one group (n = 4) was given 10 mg/kg dexamethasone im and a second group (n = 4) 2 mg/kg im.

Analysis

Plasma samples were analyzed using a reverse phase HPLC method modified from a procedure described by Plezia and Berens (1985). Methylprednisolone (2 µg) was added to 0.1-0.2 ml of plasma as an internal standard. After dilution with deionized water to a total volume of 1.2 to 1.3 ml, samples were extracted using a C_{18} cartridge (Fischer, Itasca, IL) connected to a vacuum manifold (Vac-Elut; Analytichem International, Habor City, CA). The sample containers were rinsed with 2.0 ml of 20% acetonitrile solution and the rinse was added to the C_{18} column. Vacuum was applied for 2 to 3 min after all solvent had entered the cartridge in order to remove residual water. The C_{18} cartridge was removed and attached to the top of a silica cartridge (Analytichem) and the combined cartridges were placed back on the vacuum manifold. The drugs were then eluted with 2.5 ml of acetone-chloroform (3:1). The eluent was concentrated in a "Meyer N-Evap" evaporator (Organomation, South Berlin, MA) at 36°C under nitrogen. The concentrated residue was redissolved in 0.2 ml of 20% acetonitrile before HPLC analysis.

The HPLC system consisted of a Series-10 isocratic pump and a model LC-85 ultraviolet detector (Perkin-Elmer, Norwalk, CT) set at 254 nm. A 125 mm x 4 mm, 5 µ particle size C18 column (Whatman, Clifton, NJ) was used for separations in a mobile phase of 35% acetonitrile at a flow rate of 1.1 ml/min. A model 3551-40 autosampler (Hitachi, Japan) was used to inject standards and sample extracts. Peak heights were recorded using a model 3390 integrator (Hewlett Packard, Avondale, PA).

Data Analysis

The time (t) versus plasma concentration (C_p) data for each animal were plotted on semilogarithmic paper and initial estimates of the distribution rate constant (α), elimination rate constant (β), intercept on y axis as related to alpha (A) and intercept on y axis as related to beta (B), were obtained graphically (Notari, 1975). The SAAM 27 non-linear least squares regression program (Berman and Weiss, 1977) was used to fit these estimates to both mono- and biexponential equations, i.e., one and two compartment open models, respectively, for each animal. The observed C_p values were weighted by their inverse square. Akaike's information criterion (AIC) (Yamaoka et al., 1978) was used to determine the most appropriate model for each animal. The model with the smallest AIC was deemed the most appropriate.

The area under the plasma concentration-time curve (AUC), area under the first moment concentration-time curve (AUMC), apparent volume of distribution (Vd_{area}), and the volume of distribution at steady state (Vd_{ss}), were calculated using final values for A, B, α, and β according to Gibaldi and Perrier (1982). The area under the plasma concentration-time curve, AUC(T), and the area under the moment plasma concentration-time curve, AUMC(T), were also estimated by the model-independent trapezoidal method (Gibaldi and Perrier, 1982; Riegelman and Collier, 1980). The model independent estimates of $Vd_{area}(T)$, $Vd_{ss}(T)$ and CL(T) were calculated by substituting AUC(T) and AUMC(T) in the appropriate equations.

Pharmacokinetic parameters from each experiment were compared using a paired t-test since animals were dosed in a crossover fashion. The parameters α and ka were not analyzed statistically because they were inadequately delineated due to insufficient blood sampling in the first 40 min.

Results

Average plasma concentrations versus time for each experiment are presented in Figures I.40-I.42. Within each study, pharmacokinetic parameters (Vd_{area} Vd_{ss}, Cl, AUC, and AUMC) calculated by model-independent trapezodial rule (T) did not differ statistically from estimates using model-dependent methods.

Experiment 1. 10 mg/kg iv vs 10 mg/kg im.

Semilog plots of average dexamethasone plasma concentration versus time following iv and im injection appeared to have similar slopes (Fig. I.40). Most animals were best described by 2-compartment open models as determined by Akaike's Information Criterion; rat 9x was best fit by a 1-compartment model (Table I.37). Means of pharmacokinetic parameters from iv and im administration did not differ significantly ($p < 0.05$). The estimated fraction of the dose absorbed (absolute bioavailability, F after im administration was 1.127 ± 0.254; no outliers were detected (Cook, 1977). Since F cannot exceed 1 and the estimated confidence interval includes 1, absorption can be considered to have been 100%.

Experiment 2. 10 mg/kg iv vs 2 mg/kg iv

Figure I.41 is a semilog plot of average dexamethasone plasma concentrations versus time following injection of 10 mg/kg and 2 mg/kg iv; slopes were similar for both doses. Dexamethasone was not detected in plasma samples taken after 10 h for the 2 mg/kg dose. Plasma concentrations for 9 of 11 animals dosed at 10 mg/kg iv, and 5 of 11 animals dosed at 2 mg/kg iv were best described by a 1-compartment model and a 2-compartment model described the others (Table I.38). Most parameters did not differ significantly between doses; however, Vd_{SS} and $Vd_{SS}(T)$ differed ($p < 0.03$).

Experiment 3. 10 mg/kg im vs 2 mg/kg im

Plasma concentrations of dexamethasone after im administration of 10 and 2 mg/kg are shown in a semilog plot in Figure I.42. As in Experiment 2, dexamethasone was not detected in the plasma after 10 h for the 2 mg/kg dose. Absorption of dexamethasone after im administration was very rapid. Dexamethasone plasma concentrations peaked between 5 and 40 min after the 10 mg/kg dose and by 5 min after the 2 mg/kg dose. The first-order absorption rate (ka) was poorly estimated because there were not enough samples taken within the first 40 min to delineate the absorption phase.

A 2-compartment model fit the data for the 8 animals dosed at 2 mg/kg. In contrast, only 2 of the 8 animals dosed at 10 mg/kg were best fit by a 2-compartment model (Table I.39). Most parameters did not differ between doses; only β differed significantly ($p = 0.001$). However, because of the large standard deviations associated with Vd_{area} and Vd_{SS} at the 2 mg/kg dose, we may have committed a type II error (i.e., accepted a false null hypothesis since the power of the test was < 0.5).

Discussion

The pharmacokinetics of dexamethasone have been studied in several species using RIA or RP-HPLC. The latter method is preferred for analysis of dexamethasone in biological fluids since it is sensitive and specific (Tsuei et al., 1978; Cham et al., 1980; Cairns et al., 1983; Plezia and Berens, 1985). These features allowed us to use rats in crossover experiments to examine for dose-dependent kinetics of dexamethasone given iv and im and to determine drug bioavailability after im administration. Due to the small body size of rats and the need for repetitive sampling, only 0.1 to 0.2 ml samples of plasma were available for HPLC analysis. This resulted in higher minimum detection limits in this study compared to previously reported HPLC methods (Cham et al., 1980; Plezia and Berens, 1985).

It was assumed that dexamethasone sodium phosphate administered iv to the rat would be rapidly hydrolyzed to dexamethasone alcohol. Following Tsuei et al. (1979), it was further assumed that the disposition kinetics of dexamethasone alcohol would not be significantly affected by this process. In our experiments, the dexamethasone alcohol plasma concentration was at a maximum within 5 min after iv administration of dexamethasone sodium phosphate. This agrees with reports that plasma concentrations peaked within 5 min (Tsuei et al., 1979) or 10 min (Hare et al., 1975) after iv dosing in humans.

While previous workers had studied the disposition of iv administered dexamethasone sodium phosphate, the disposition of the drug following im administration was unknown. Our first experiment showed that dexamethasone alcohol was completely available after im administration of dexamethasone sodium phosphate (10 mg/kg dexamethasone alcohol equivalent). The drug was rapidly absorbed with the peak plasma concentration occurring between 5 and 40 min postdosing. Furthermore, the pharmacokinetic parameters for elimination, clearance, and distribution following im and iv administration of dexamethasone at 10 mg/kg were statistically indistinguishable (Table I.39). Therefore, the route of administration had no discernible effect on the distribution and elimination of dexamethasone from the body.

Varma and Mulay (1980) found a trend toward a longer elimination half-life for dexamethasone as the dose decreased from 3 to 1 mg/kg. However, because of the relatively large coefficient of variation (25 to 50%), this trend was not statistically significant. We therefore carried out studies to detect dose-dependent kinetics of dexamethasone administered iv and im at either 10 mg/kg or 2 mg/kg. General evidence of dose-dependent kinetics associated with an increased dose would be a nonlinear scaling of AUC (i.e., $AUC_{10} \neq 5\ AUC_2$) and a smaller ß. With drugs that are highly plasma protein bound, drug sequestration in the central compartment results in a smaller Vd_{SS} as the drug dose decreases. In contrast, highly tissue protein bound drugs sequester in the periphery, producing a higher Vd_{SS} with decreasing dose. Therefore, Vd_{SS} is often used to distinguish the site of binding (Gibaldi and Perrier, 1982).

In study 2 (iv administration), the difference in the mean (± S.D.) value of ß for 2 mg/kg iv (0.2534 ± 0.0549 h^{-1}) and 10 mg/kg iv (0.2869 ± 0.0673 h^{-1}) doses was not statistically different ($p = 0.295$). In study 3 (im administration), the difference in the mean value of ß for 2 mg/kg im (0.209 ± 0.085 h^{-1}) and 10 mg/kg im (0.294 ± 0.123 h^{-1}) doses was significant ($p = 0.001$). If the differences in ß at the 2 doses reflect a real biological difference, this would be evidence of dose-dependent kinetics. Further evidence was given in Experiment 2 by significantly higher values of Vd_{SS} and $Vd_{SS}(T)$ ($p < 0.03$) at the low dose. If this increase in Vd_{SS} was biologically significant, it would suggest a capacity-limited tissue binding of dexamethasone. However, no change in Vd_{SS} was found in study 3. If the significant results in study 2 are truly reflective of dose-dependent kinetics, and the nonsignificant results in study 3 are failures to reject a false null hypothesis (Type 2 error), AUC should also scale nonlinearly with dose. This was not found. In both experiments, AUC was proportional to dose indicating that disposition followed first-order kinetics. Evidence for dose-dependency was therefore inconsistent, and we conclude that, as in other studies, the evidence was weak at best.

Study 3 (Table I.39), like study 1, showed that the absorption of dexamethasone after im injection was very rapid with the peak concentration (Cp) occurring within 5 min following the 2 mg/kg dose and between 5 and 40 min following the 10 mg/kg dose. Since both doses contained the same dexamethasone concentration, different volumes were administered. Therefore, the differences in the time to maximum absorption may reflect the larger surface area to volume ratio at 2 mg/kg which would increase the relative amount of drug in direct contact with perfused tissue.

In studies 2 and 3, elimination half-lives of dexamethasone in rats given 2 mg/kg and 10 mg/kg iv were 2.74 h (n = 11) and 2.61 h (n = 20). Varma and Mulay (1980), using the RIA method, reported comparable elimination half-lives in rats of 5.4 h (n = 6), 3.9 h (n = 7), and 3.6 h (n = 5) after iv administration of 1, 2, and 3 mg/kg, respectively. Further, our estimates for Vd_{area} (1.176 ± 0.413 l/kg) and Cl (0.2837 ± 0.074 l/kg/h) after adminstration of 2 mg/kg iv (Table 4) were similar to theirs; 0.965 ± 0.392 l/h and 0.180 ± 0.048 l/kg/h, respectively. Thus, our experiments gave no evidence that the RP-HPLC and RIA methods gave different estimates of pharmacokinetic parameters.

To use the rat as a model for the therapeutic efficacy of dexamethasone in humans, it is necessary to determine the relationship between the pharmacokinetics of the drug in the two species. Since pharmacokinetic information is available for several species, this description can be accomplished by establishing allometric relationships (Mordenti, 1985a,b). The rate of plasma clearance after iv administration is similar in rats (0.266 l/kg/h, N = 20) and humans (0.18 to 0.24 l/kg/h) suggesting that the rates of metabolism of dexamethasone are comparable. Clearance rates (iv) in cattle, dogs, and horses are 0.145, 0.384, and 0.774 l/kg/h, respectively. The significant allometric relationship was: CL (l/h) = 0.255 W ($p < 0.05$). The volume of distribution (Vd_{area}) was about the same (0.9 to 1.0 l/kg) in all species.

This research has shown that the rat may be an acceptable model to study therapeutic aspects of dexamethasone in humans because of the similarity in pharmacokinetic parameters between humans and rats. Furthermore, this research suggests, but does not decidedly demonstrate, dose-dependent pharmacokinetics.

Acknowledgement

The authors express their appreciation to M. Busse, T. Keferlis, B. Kindler, R. Manuel, and Drs. R. Lovell and V. Pang for their technical assistance.

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Table I.37 Experiment 1: Pharmacokinetic parameters for dexamethasone in rat plasma following iv administration at 10 mg/kg body weight.

Animal		1	2	3	4	5X	6X	7X	8X	9X	Mean	SD
Weight	kg	0.256	0.270	0.295	0.330	0.250	0.239	0.232	0.253	0.225	0.2611	0.0332
Dose	mg/kg	9.58	9.64	9.78	9.65	10.24	9.83	10.1	9.61	10.4	9.869	0.3098
β	h^{-1}	0.103	0.288	0.275	0.248	0.236	0.301	0.235	0.162	0.305	0.2415	0.0648
B	µg/ml	0.806	10.2	10.4	9.06	9.28	10.4	8.16	7.12	11.1	8.497	3.139
α	h^{-1}	0.365	5.88	3.67	0.845	0.493	2.98	0.982	1.42		2.079	1.942
A	mg	11.9	4.95	8.48	4.35	3.81	9.68	5.40	8.70		7.156	2.926
AUC	µg/ml/h	40.4	36.3	40.1	41.6	47.1	37.7	40.3	45.2	36.4	40.55	3.692
AUC(T)	µg/ml/h	42.2	37.1	41.8	42.5	47.8	37.9	41.3	48.2	38.4	41.89	3.985
AUMC	µg-h^2/ml	164	123	138	153	182	116	154	218	119	152.1	33.41
AUMC(T)	µg-h^2/ml	179	122	141	153	184	115	154	222	116	154.9	35.82
Vd_{area}	l/kg	2.30	0.922	0.887	0.934	0.922	0.867	1.07	1.17	0.941	1.111	0.4551
$Vd_{area}(T)$	l/kg	2.20	0.902	0.852	0.913	0.907	0.863	1.04	1.10	0.892	1.074	0.4292
VD_{ss}	l/kg	0.969	0.902	0.840	0.852	0.843	0.800	0.954	1.03	0.941	0.9033	0.0751
$VD_{ss}(T)$	l/kg	0.961	0.857	0.788	0.814	0.825	0.791	0.907	0.920	0.825	0.8541	0.0614
CL	l/kg/h	0.237	0.266	0.244	0.232	0.218	0.261	0.250	0.213	0.287	0.2452	0.0237
CL(T)	l/kg/h	0.227	0.260	0.234	0.227	0.214	0.260	0.244	0.200	0.272	0.2375	0.0236
AIC_1		-20.8	-17.1	-21.1	-42.6	-16.5	-35.6	-19.4	5.06	-29.9		
AIC_2		-34.8	-33.8	-28.5	-47.9	-18.0	-48.9	-38.0	-0.396	-28.7		
Model		2	2	2	2	2	2	2	2	1		

Table I.37 (continued) Experiment 1: Pharmacokinetic parameters for dexamethasone in rat plasma following im administration at 10 mg/kg body weight.

Animal		1X	2X	3X	4X	5	6	7	8	9	Mean	SD
Weight	kg	0.240	0.238	0.267	0.288	0.264	0.265	0.260	0.277	0.243	0.2602	0.0171
Dose	mg/kg	10.7	10.1	9.80	10.4	11.8	9.85	9.84	9.88	9.95	10.24	0.6418
ß	h^{-1}	0.087	0.344	0.234	0.229	0.331	0.295	0.259	0.136	0.212	0.2361	0.0849
B	µg/ml	1.71	12.9	11.2	9.7	13.7	14.1	9.07	0.801	10.81	9.341	4.895
α	h^{-1}	0.252			0.888			0.703	0.332	0.600	0.5551	0.2628
A	µg/ml	9.23			4.31			6.48	12.8	4.36	7.427	3.598
ka	h^{-1}	21.2	14.7	13.0	10.2	21.1	19.5	10.4	21.6	12.2	16.00	4.838
AUC	µg/ml/h	55.8	36.7	47.1	46.1	40.7	47.2	42.7	43.7	57.1	46.34	6.640
AUC(T)	µg/ml/h	59.0	38.4	47.5	46.7	41.3	47.7	42.1	45.8	57.7	47.45	6.980
AUMC	µg-h^2/ml	373	113	205	192	166	163	148	169	254	198.0	76.42
AUMC(T)	µg-h^2/ml	455	115	204	190	165	162	147	187	252	208.8	99.77
F		1.24	0.97	1.17	1.03	0.75	1.25	1.09	0.941	1.65	1.121	0.254
F(T)		1.26	0.990	1.13	1.02	0.752	1.26	1.05	0.925	1.58	1.107	0.238
Vd_{area}	l/kg	2.21	0.772	0.891	0.984	0.656	0.708	0.889	1.57	0.823	1.055	0.507
$Vd_{area}(T)$	l/kg	2.09	0.756	0.883	0.969	0.646	0.701	0.902	1.47	0.815	1.025	0.465
VD_{ss}	l/kg	1.28	0.817	0.907	0.936	0.888	0.718	0.798	0.823	0.773	0.8818	0.163
$VD_{ss}(T)$	l/kg	1.39	0.782	0.887	0.904	0.859	0.703	0.815	0.818	0.754	0.8793	0.202
CL	l/kg/h	0.191	0.266	0.208	0.225	0.218	0.209	0.230	0.213	0.174	0.2148	0.026
CL(T)	l/kg/h	0.181	0.260	0.207	0.222	0.214	0.207	0.234	0.200	0.173	0.2106	0.026
AIC_1		-1.81	-18.0	-32.9	-18.0	-20.2	-39.5	-26.6	-28.9	-18.2		
AIC_2		-4.04	-14.0	-29.3	-21.8	-17.1	-34.7	-34.5	-30.9	-25.2		
Model		2	1	1	2	1	1	2	2	2		

I.38 Experiment 2: Pharmacokinetic parameters for dexamethasone in rat plasma following im administration at 10 mg/kg of body weight.

nal		10	11	12	13	14X	15X	16X	17X	18X	19X	20X	Mean	SD
t	kg	0.246	0.273	0.304	0.300	0.228	0.228	0.242	0.245	0.260	0.302	0.279	0.2643	0.0290
	mg/kg	10.8	9.76	11.4	9.89	10.4	10.1	9.97	10.1	9.78	10.1	10.11	10.23	0.4976
	h^{-1}	0.109	0.301	0.263	0.303	0.368	0.337	0.323	0.289	0.321	0.285	0.257	0.2869	0.0673
	µg/ml	1.24	10.33	11.74	14.1	13.7	11.9	10.9	11.5	12.8	12.5	11.7	11.12	3.463
	h^{-1}	0.411	1.89										1.148	1.043
	µg/ml	15.3	4.51										9.925	7.652
	µg/ml/h	48.8	36.7	44.6	46.4	37.3	35.1	33.6	39.7	39.8	43.9	45.38	41.04	5.041
)	µg/ml/h	53.3	37.7	46.6	47.3	37.4	37.8	34.2	39.7	40.9	47.3	46.9	42.65	5.929
	µg-h^2/ml	196	115	170	153	101	105	104	137	124	154	177	139.6	32.67
(T)	µg-h^2/ml	217	115	184	155	100	113	103	138	131	167	181	145.8	38.11
ea	l/kg	2.05	0.883	0.973	0.703	0.759	0.852	0.919	0.880	0.766	0.811	0.868	0.9507	0.3714
ea(T)	l/kg	1.87	0.859	0.931	0.689	0.757	0.796	0.902	0.881	0.746	0.753	0.839	0.9111	0.3268
	l/kg	0.894	0.835	0.973	0.703	0.759	0.852	0.919	0.880	0.766	0.811	0.868	0.8416	0.0782
(T)	l/kg	0.827	0.788	0.968	0.689	0.746	0.800	0.883	0.887	0.765	0.754	0.829	0.8123	0.0783
	l/kg/h	0.222	0.266	0.256	0.213	0.280	0.287	0.297	0.255	0.246	0.231	0.223	0.2522	0.0283
)	l/kg/h	0.203	0.259	0.245	0.209	0.279	0.268	0.292	0.255	0.239	0.214	0.215	0.2435	0.0300
		-4.5	-14.2	-13.0	-38.9	-20.2	0.249	-37.2	-30.5	-33.3	-22.4	-14.9		
		-7.47	-19.6	-9.43	-34.9	-18.2	4.25	-36.6	-28.8	-29.3	-18.4	-12.3		
		2	2	1	1	1	1	1	1	1	1	1		

able I.38 (continued) Experiment 2: Pharmacokinetic parameters for dexamethasone in rat plasma following iv administration at 2 mg/kg of body weight.

Animal		10X	11X	12X	13X	14	15	16	17	18	19	20	Mean	SD
leight	kg	0.233	0.240	0.280	0.270	0.235	0.240	0.266	0.256	0.280	0.326	0.288	0.2649	0.0283
ose	mg/kg	1.97	1.97	2.24	2.23	2.28	2.24	1.95	1.97	2.22	2.19	1.69	2.087	0.1857
$_{10}/D_2$	---	5.50	4.95	5.10	4.44	4.57	4.52	5.11	5.13	4.41	4.64	5.97	4.939	0.4892
	h^{-1}	0.293	0.232	0.239	0.248	0.269	0.126	0.310	0.311	0.222	0.229	0.310	0.2534	0.0549
	μg/ml	2.03	1.87	0.96	1.57	2.39	0.584	1.49	2.07	2.11	1.57	2.62	1.751	0.6006
	h^{-1}			1.76		2.67	0.55	1.57		1.98	0.63		1.527	0.8169
	μg/ml			1.56		1.39	2.49	1.48		0.759	1.49		1.528	0.5544
UC	μg/ml/h	5.92	8.09	4.91	6.32	9.43	9.13	5.75	6.65	9.92	9.23	8.45	7.709	1.679
UC(T)	μg/ml/h	7.26	8.29	5.05	6.55	9.69	9.44	5.96	6.81	10.1	9.60	8.60	7.936	1.705
UMC	μg-h^2/ml	23.6	35.0	17.3	25.5	33.4	44.9	16.1	21.4	43.3	33.7	27.3	29.22	9.683
UMC(T)	μg-h^2/ml	27.3	36.7	17.0	26.1	33.0	45.4	16.2	22.1	43.0	34.2	27.1	29.83	9.632
UC[a]	μg/ml/h	38.1	40.0	25.0	28.1	43.1	41.3	29.4	34.2	43.7	42.8	50.4	37.82	7.792
UC(T)[a]	μg/ml/h	39.9	41.0	25.7	29.1	44.3	42.7	30.4	35.0	44.4	44.5	51.3	38.93	7.887
UMC[a]	μg-h^2/ml	129	173	88.3	113	153	203	82.3	109	191	156	163	142.0	40.36
UMC(T)[a]	μg-h^2/ml	149	181	86.7	116	151	205	82.7	114	189	158	161	145.1	40.70
$/d_{area}$	l/kg	0.971	1.06	1.91	1.42	0.900	1.95	1.10	0.950	1.01	1.04	0.647	1.176	0.4130
$/d_{area}(T)$	l/kg	0.927	1.03	1.86	1.37	0.875	1.88	1.06	0.929	0.996	0.997	0.635	1.141	0.3993
$/D_{ss}$	l/kg	0.971	1.06	1.61	1.42	0.855	1.21	0.951	0.950	0.976	0.867	0.647	1.046	0.2722
$/D_{ss}(T)$	l/kg	1.02	1.06	1.50	1.36	0.802	1.14	0.891	0.939	0.942	0.812	0.620	1.007	0.2518
L	l/kg/h	0.285	0.244	0.456	0.353	0.242	0.245	0.340	0.296	0.224	0.237	0.200	0.2837	0.0743
L(T)	l/kg/h	0.272	0.238	0.444	0.340	0.235	0.237	0.328	0.289	0.221	0.228	0.197	0.2753	0.0717
IC$_1$	---	-35.4	-21.3	-15.7	-31.7	-31.0	-25.3	-20.2	-27.5	-35.4	-28.1	-42.8		
IC$_2$	---	-15.3	-20.2	-19.5	-29.6	-34.1	-41.2	-26.1	-26.7	-47.5	-31.7	-39.2		
lodel		1	1	2	1	2	2	2	1	2	2	1		

[a]Parameter scaled to the appropriate $Dose_{10}$.

Table I.39 Experimemt 3: Pharmacokinetic parameters for dexamethasone in rat plasma following im administration at 10 mg/kg of body weight.

Animal		21	22	23	24	25X	26X	27X	28X	Mean	SD
Weight	kg	0.266	0.266	0.261	0.264	0.247	0.252	0.253	0.234	0.2554	0.0112
Dose	mg/kg	9.62	9.59	9.89	9.58	10.3	9.51	9.42	10.4	9.798	0.3852
ß	h^{-1}	0.263	0.312	0.302	0.278	0.290	0.325	0.213	0.370	0.2941	0.0462
B	µg/ml	9.41	14.4	16.2	12.2	14.8	17.0	5.97	20.2	13.77	4.508
α	h^{-1}	1.27						0.383		0.8398	0.6255
A	µg/ml	5.47						8.267		6.869	1.978
ka	h^{-1}	14.4	20.4	22.5	20.2	11.8	3.19	25.5	1.67	14.96	8.865
AUC	µg/ml/h	39.0	45.5	53.0	43.1	49.8	47.1	48.9	42.6	46.12	4.501
AUC(T)	µg/ml/h	39.9	47.0	48.9	43.5	52.6	47.7	49.7	42.5	46.47	4.194
AUMC	µg-h^2/ml	139	148	178	157	176	160	187	141	160.7	18.00
AUMC(T)	µg-h^2/ml	138	148	170	161	187	166	188	138	162.3	19.60
Vd_{area}	l/kg	0.937	0.676	0.618	0.799	0.716	0.622	0.907	0.662	0.7422	0.1252
$Vd_{area}(T)$	l/kg	0.916	0.654	0.670	0.792	0.678	0.614	0.891	0.664	0.7349	0.1159
VD_{ss}	l/kg	0.880	0.686	0.626	0.810	0.734	0.685	0.738	0.809	0.7460	0.0826
$VD_{ss}(T)$	l/kg	0.838	0.645	0.706	0.817	0.698	0.694	0.719	0.800	0.7394	0.0694
CL	l/kg/h	0.247	0.211	0.187	0.222	0.207	0.202	0.193	0.245	0.2142	0.0223
CL(T)	l/kg/h	0.240	0.201	0.203	0.220	0.196	0.199	0.190	0.246	0.2123	0.0210
AIC_1		-8.96	-18.7	-9.37	-17.3	-23.2	-31.4	-25.2	-32.4		
AIC_2		-14.3	6.01	-6.45	-15.7	-21.2	-27.4	-31.8	-28.4		
Model		2	1	1	1	1	1	2	1		

Table I.39 (continued) Experiment 3: Pharmacokinetic parameters for dexamethasone in rat plasma following im administration at 2 mg/kg of body weight.

Animal		21X	22X	23X	24X	25	26	27	28	Mean	SD
Weight	kg	0.248	0.252	0.246	0.247	0.274	0.266	0.274	0.245	0.2565	0.0127
Dose	mg/kg	2.39	2.45	2.32	2.50	1.98	1.95	1.88	1.89	2.169	0.2672
D_{10}/D_2	---	4.04	3.92	4.27	3.83	5.21	4.89	5.01	5.52	4.585	0.6480
ß	h^{-1}	0.123	0.243	0.281	0.220	0.182	0.283	0.053	0.285	0.2086	0.0846
B	µg/ml	0.804	1.41	1.90	2.03	0.954	2.17	0.250	2.42	1.494	0.7651
α	h^{-1}	0.546	0.749	1.25	1.37	0.607	1.59	0.529	4.11	1.343	1.189
A	µg/ml	2.53	2.48	1.29	2.56	2.49	1.75	2.81	1.18	2.136	0.634
ka	h^{-1}	341	723	999	154	69.2	999	253	999	567.2	405.7
AUC	µg/ml/h	11.2	9.11	7.82	11.1	9.30	8.79	10.0	8.78	9.505	1.170
AUC(T)	µg/ml/h	11.9	9.30	7.96	11.2	9.55	8.90	10.3	8.75	9.738	1.322
AUMC	µg-h^2/ml	61.8	28.3	25.0	43.3	35.6	27.9	98.6	29.8	43.79	25.15
AUMC(T)	µg-h^2/ml	70.2	28.5	25.2	42.4	35.9	27.8	105	29.4	45.56	28.15
AUC[a]	µg/ml/h	45.1	35.7	33.4	42.5	48.4	43.0	50.1	48.4	43.31	6.087
AUC(T)[a]	µg/ml/h	47.9	36.4	34.0	43.1	49.7	43.5	51.8	48.3	44.33	6.373
AUMC[a]	µg-h^2/ml	249	111	107	166	186	137	494	164	201.7	126.4
AUMC(T)[a]	µg-h^2/ml	283	111	108	162	187	136	526	162	209.5	139.6
Vd_{area}	l/kg	1.74	1.11	1.06	1.03	1.17	0.784	3.53	0.756	1.397	0.9149
$Vd_{area}(T)$	l/kg	1.64	1.08	1.04	1.01	1.14	0.774	3.42	0.758	1.350	0.8772
VD_{ss}	l/kg	1.18	0.836	0.948	0.882	0.817	0.703	1.85	0.733	0.9940	0.3766
$VD_{ss}(T)$	l/kg	1.19	0.806	0.920	0.838	0.781	0.683	1.85	0.727	0.9744	0.3873
CL	l/kg/h	0.214	0.269	0.296	0.226	0.213	0.221	0.188	0.216	0.2303	0.0349
CL(T)	l/kg/h	0.201	0.264	0.291	0.222	0.208	0.219	0.182	0.216	0.2253	0.0352
AIC_1		-17.7	-21.2	-25.1	-20.0	-26.6	-36.1	-39.8	-36.9		
AIC_2		-19.2	-25.6	-33.1	-39.2	-44.2	-41.8	-40.5	-43.7		
Model		2	2	2	2	2	2	2	2		

[a]Parameter scaled to the appropriate $Dose_{10}$.

Figure I.40 Experiment 1: Semilogarithmic plot of averaged dexamethasone plasma concentrations in 9 rats following iv (□) or im (■) administration of 10 mg/kg of dexamethasone. Points were experimentally determined (± 1 SD).

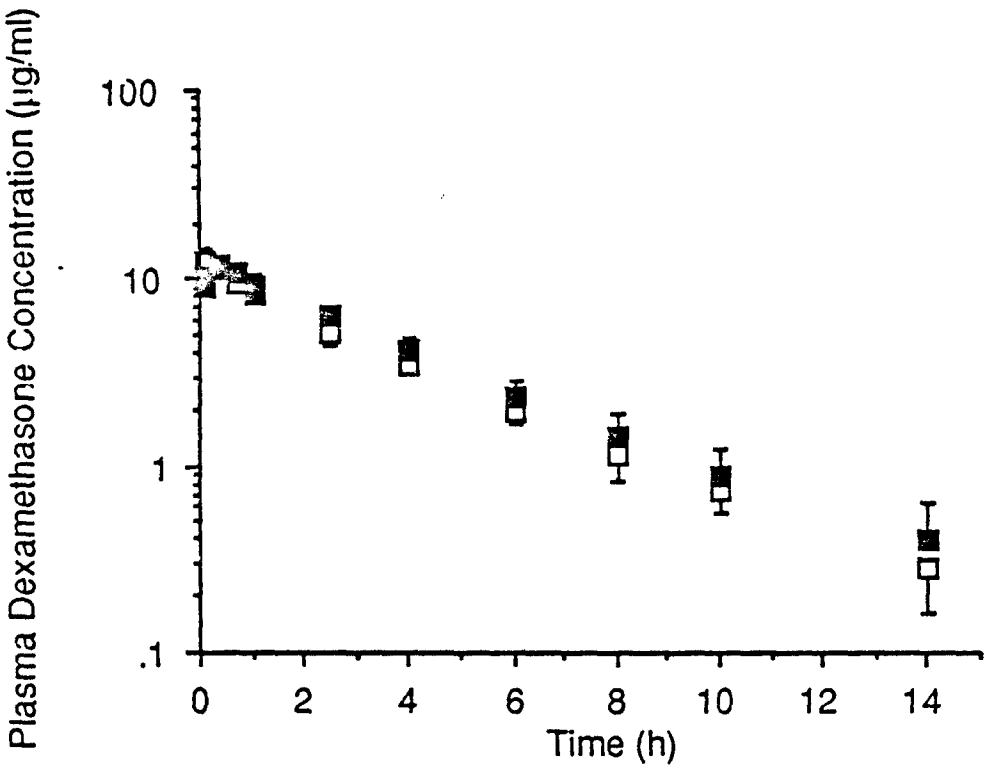


Figure I.41 Experiment 2: Semilogarithmic plot of averaged dexamethasone plasma concentrations in 11 rats following iv administration of 10 mg/kg (■) or 2 mg/kg (□) of dexamethasone. Points were experimentally determined (± 1 SD).

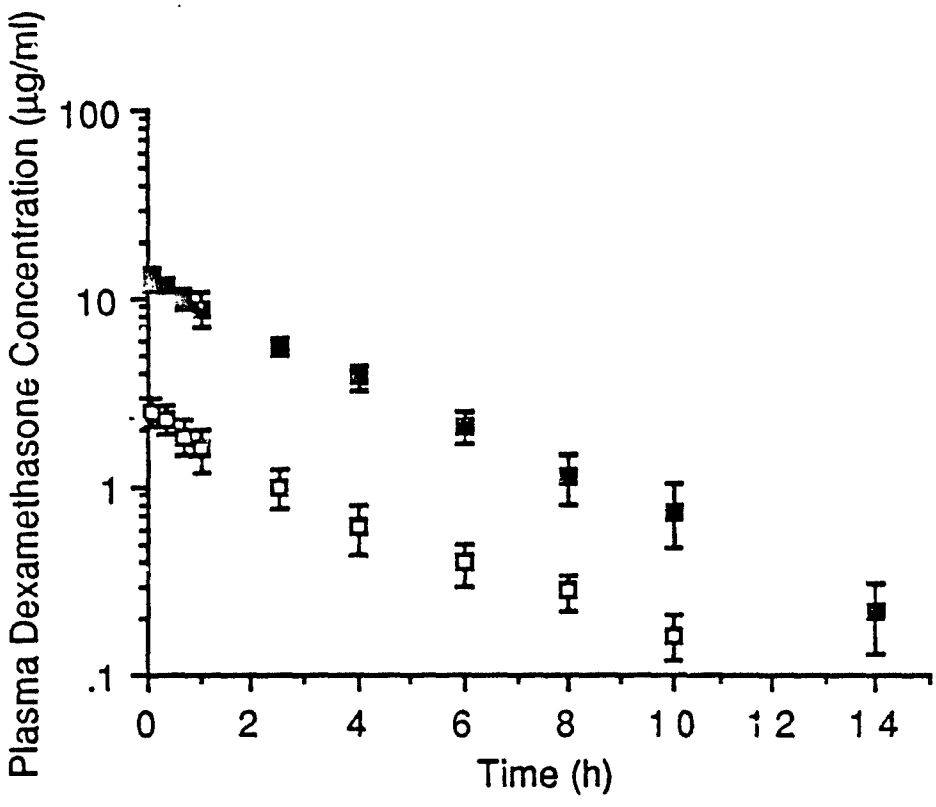
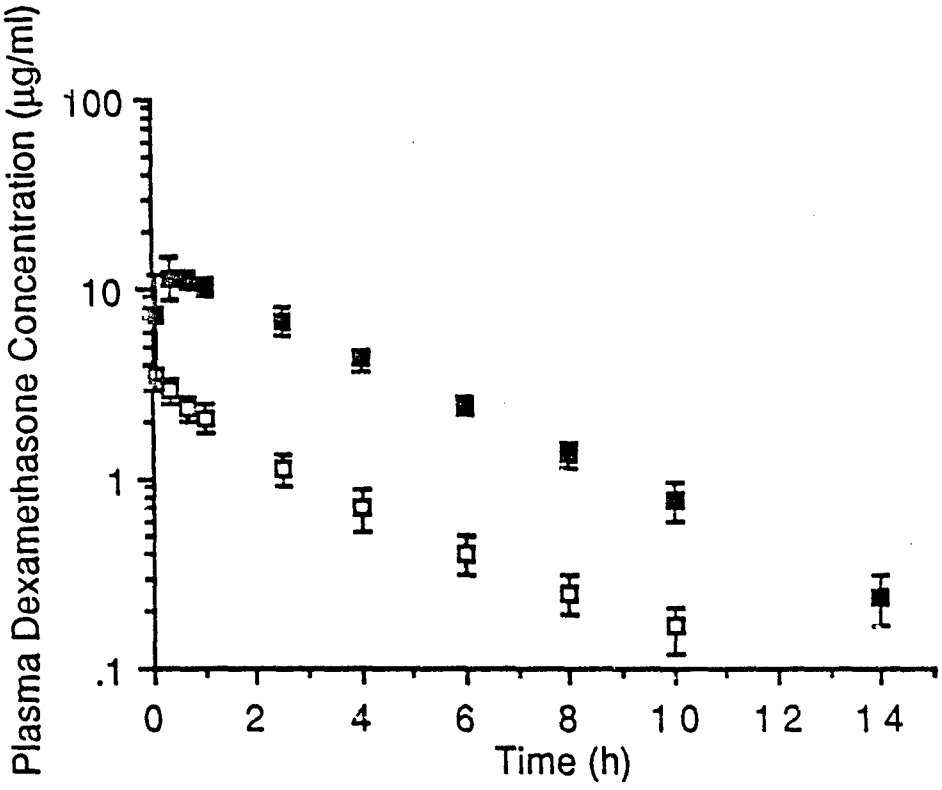


Figure I.42 Experiment 3: Semilogarithmic plot of averaged dexamethasone plasma concentration in 8 rats following im administration of 10 mg/kg (■) or 2 mg/kg (□) of dexamethasone. Points were experimentally determined (± 1 SD).



APPENDIX

The 1-compartment open model after iv administration is given by Eq. 1 and the 2-compartment model by Eq. 2:

$$C_p = Be^{-\beta t} \quad (1)$$
$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \quad (2)$$

For im administration, the 1- and 2-compartment open models with a first order rate constant of absorption are:

$$C_p = Be^{-\beta t} - Be^{-kat} \quad (3)$$
$$C_p = Ae^{-\alpha t} + Be^{-\beta t} - (A+B)e^{-kat} \quad (4)$$

The AUC and AUMC for a 1-compartment model after iv administration are given by:

$$AUC_{iv} = B/\beta \quad (5)$$
$$AUMC_{iv} = B/\beta^2 \quad (6)$$

For a 2-compartment model, the corresponding equations are:

$$AUC_{iv} = A/\alpha + B/\beta \quad (7)$$
$$AUMC_{iv} = A/\alpha^2 + B/\beta^2 \quad (8)$$

For a 1-compartment model after im administration with a first order rate constant of absorption, the parameters are:

$$AUC_{im} = B/\beta - B/ka \quad (9)$$
$$AUMC_{im} = B/\beta^2 - B/(ka)^2 \quad (10)$$

For a 2-compartment model, the equations are:

$$AUC_{im} = A/\alpha + B/\beta - (A+B)/ka \quad (11)$$
$$AUMC_{im} = A/(\alpha)^2 + B/(\beta)^2 - (A+B)/(ka)^2 \quad (12)$$

The apparent volume of distribution using the area under the curve (Vd_{area}) and the apparent volume of distribution at steady state (Vd_{ss}) were estimated using:

$$Vd_{area} = Dose/(AUC \times \beta) \quad (15)$$
$$Vd_{ss} = (Dose)(AUMC)/AUC^2 \quad (16)$$

The area under the curve by the trapezoidal method was calculated by adding up adjacent trapezoids under the curve and adding an estimate of the terminal portion of the curve with the formula:

$$[(C_n + C_{n-1})/2]\delta + C_z/\beta \quad (17)$$

where $\delta = t_n - t_{n-1}$, n-1, and n represent adjacent data point times, and C_z is the concentration at the last data point time, t_z. The area under the moment curve is similarly estimated by the trapezoidal method using the correction factor given by:

$$[(t_nC_n + t_{n-1}C_{n-1})/2]\delta + t_zC_z/\beta + C_z/\beta^2 \quad (18)$$

To compare AUC and AUMC between the 2 doses, the estimates for the 2 mg/kg dose were normalized to an equivalent dose of 10 mg/kg using:

$$AUC_{10} = (Dose_{10})(AUC_2)/Dose_2$$
$$AUMC_{10} = (Dose_{10})(AUMC_2)/Dose_2$$

Clearance (CL) was calculated as:

$$CL = Dose/AUC$$

The absolute bioavailability (F) of dexamethasone after im administration was determined using:

$$F_{ab} = [(Dose_{iv})(AUC_{im})]/[(Dose_{im})(AUC_{iv})]$$

2. The effect of acute T-2 toxicosis on the plasma disposition of dexamethasone

by
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S. P. Swanson, and W. B. Buck

Summary

The disposition of plasma dexamethasone was determined in female Sprague-Dawley rats given T-2 toxin. Dexamethasone was given intravenously (IV) or intramuscularly (IM) immediately or 1 h after rats had been given an approximate LD_{50} dose of T-2 toxin (0.75 mg/kg, IV). Plasma concentrations were compared to that of control animals dosed with dexamethasone alone. Rats given dexamethasone IV immediately or 1 h after exposure to T-2 toxin had plasma concentrations which were similar and significantly higher than controls after 2.5 h. Animals treated immediately IM had significantly ($p > 0.05$) higher plasma concentrations than controls after 8 h, while those treated after a 1 h delay had higher plasma concentrations than controls by 4 h ($p < 0.05$). In contrast, plasma dexamethasone concentrations in animals given dexamethasone IM immediately after the toxin differed from those given the delayed therapy by 1 h.

Introduction

T-2 toxin, a trichothecene mycotoxin, is a secondary metabolite produced by several species of Fusarium which has been associated with toxicosis in farm animals (Ueno, 1977). Trichothecene mycotoxins have been implicated as a component of "Yellow Rain," a purported chemical warfare agent used in Southeast Asia and Afghanistan (Haig, 1982). It has been shown that T-2 toxicosis results in cardiovascular collapse which is similar in character to circulatory shock (Lorenzana et al., 1985; Lundeen et al., 1986; Sirén and Feuerstein, 1986).

Intravenous administration of dexamethasone has been shown to be efficacious in prolonging survival and decreasing the lethality of T-2 toxin dosed mice (Fricke, 1985) and rats (Tremel et al., 1985; Shohami et al., 1987; Ryu et al., 1987). Dexamethasone given prophylactically appears to be more efficacious than when given after the onset of clinical signs (Fricke, 1985; Tremel et al., 1985; Ryu et al., 1987).

The physiologic effects of T-2 toxicosis, including impairment of the cardiovascular and other organ systems, occurs rapidly (Sirén et al., 1986). Immediate treatment with dexamethasone should lessen the detrimental effects of the toxin and be associated with more nearly normal plasma disposition of the drug. A 1 h delay in administration would be expected to result in a decreased capacity for elimination of dexamethasone and a corresponding increase in plasma concentrations relative to controls. In addition, since the pharmacokinetics of IV or IM administration of dexamethasone are statistically indistinguishable in normal animals (Wong-Pack, 1987), the route of administration should not markedly affect the disposition of dexamethasone plasma concentrations during T-2 toxicosis.

This study was undertaken to compare the disposition of dexamethasone given IV or IM to rats (Rattus norvegicus) immediately or 1 h after IV

administration of T-2 toxin. That information would then be used to determine the dosing interval for dexamethasone in multiple-dosing therapy studies, since alterations in survival rate and times should reflect in part changes in dexamethasone disposition.

Materials and Methods

Chemicals

T-2 toxin was prepared in our laboratory from extracts of Fusarium sporotrichiodes grown on rice culture. Purity was greater than 95% as determined by capillary gas chromatography of the trimethylsilyl ether derivative using a flame ionization detector. Toxin was dissolved (1 mg/ml) in a 9:1 saline (0.9%)/ethanol solution and injected into the tail vein at an approximate LD_{50} dose (0.75 mg/kg, IV).

A predetermined dose of dexamethasone sodium phosphate[a] (10 mg/kg) was drawn into a 0.5 cc disposable syringe and weighed on an analytical balance. The syringe was reweighed after dosing to determine the actual quantity administered to each animal. Doses are reported as dexamethasone alcohol; 24 mg of dexamethasone sodium phosphate is equivalent to 19.98 mg of dexamethasone alcohol.

Animals

Virus-free female Sprague-Dawley rats[b] (225 to 300 g) were maintained in transparent polycarbonate cages containing ground corncob bedding[c] in a temperature-controlled animal room at 21-24°C with 12 h lighting, for 10 d before experimentation. The animals were given a standard commercial diet[d] and tap water ad libitum.

Surgery

Rats were anesthetized with 40 to 50 mg/kg of pentobarbital[e] IP. Cannulas were surgically implanted in both the left common carotid artery and the left external jugular vein (Weeks and Davis, 1964), externalized between the shoulder blades, and sutured in place 24 to 36 h before administration of dexamethasone. After surgery, rats were housed individually for the duration of the study. The patency of the cannulas was maintained by filling them with a heparinized saline solution (500 IU/100 ml). In addition, the cannulas were flushed with 0.2 cc of saline twice daily after removal of the heparin solution. During the blood sampling period, the animals were restrained in rat holders for the first 40 min and then returned to individual cages. The remaining blood samples were collected from the animals with minimal restraint. The

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[a]Decadron, 24 mg/ml, Merck, Sharp and Dohme, West Point, PA.

[b]Harlan Sprague-Dawley, Indianapolis, IN.

[c]San-i-cel, Paxton, IL.

[d]Lab-blox, Wayne, Chicago, IL.

[e]Nembutal, Abbott, Chicago, IL.

animals were allowed free access to feed and water during the 14-h blood sampling period.

Prior to dosing with dexamethasone, the heparin solution in both cannulas was replaced with saline. Dexamethasone was injected into the jugular vein catheter or flexor muscles of the left hind leg. Serial blood samples (0.30 ml) were collected from the carotid artery into heparinized disposable plastic syringes and transferred to microtest tubes. Blood samples were immediately chilled on ice and centrifuged at 2,900 rpm for 5 min at 5 to 10°C to separate the plasma, which was stored at -20°C for 1 to 60 d before analysis. At each sampling time, the blood volume was replaced with an equal volume of saline infused into the carotid artery.

Plasma Dexamethasone Disposition Studies

Animals were randomly assigned to either dexamethasone control or T-2 dosed groups. Each therapeutic treatment was replicated three (IV) or 4 (IM) times over 14 d; the control group was not replicated.

Control animals (n = 15) were given 10 mg/kg dexamethasone IV or IM. T-2 toxin dosed animals were given dexamethasone (10 mg/kg) immediately or 1 h after the toxin. Animals with a nonfunctional arterial cannula or which did not survive the first 10 h were removed, resulting in unequal numbers of animals between groups. Blood samples were collected predosing (during surgery) and at 5, 20, and 40 min and 1, 2.5, 4, 6, 8, 10, and 14 h following dexamethasone administration.

Chemical Analysis

Plasma dexamethasone concentrations were determined using a reverse phase HPLC method modified from a procedure described by Plezia and Berens.[11] Methylprednisolone (2 µg) was added as an internal standard. Plasma samples (0.1 to 0.2 ml) were initially extracted using a C_{18} cartridge[f] connected to a vacuum manifold.[g] After dilution with deionized water to a total volume of 1.2 to 1.3 ml, samples were applied to the C_{18} column. The sample containers were then rinsed with 2.0 ml of 20% acetonitrile and the rinses added to the C_{18} column. Vacuum was continued to remove residual water. The C_{18} cartridge was then removed and attached to the top of a preconditioned silica cartridge[g] and the compounds eluted with 2.5 ml of acetone-chloroform (3:1). The eluate was concentrated in a "Meyer N-Evap" evaporator[h] at 36°C under nitrogen. The residue was redissolved in 0.2 ml of a 20% acetonitrile solution for injection into the reverse phase HPLC system.

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[f]Fisher Scientific, Itasca, IL.

[g]Analytichem International, Harbor City, CA.

[h]Organomation, South Berlin, MA.

The HPLC system consisted of an isocratic pump,[i] autosampler,[j] integrator,[k] and an ultraviolet detector set at 254 nm.[l] A 125 mm x 4 mm id, 5 μ particle size C_{18} column[m] was used for separations with a mobile phase of 35% acetonitrile and a flow rate of 1.1 ml/min. One hundred microliter sample and standard volumes were injected by the autosampler. Peak heights were measured with the reporting integrator.

Data Analysis

Analysis of covariance (Winer, 1971) (ANCOVA) with time as the covariate was used to determine differences in plasma concentrations of dexamethasone among control and treated groups. Post-hoc linear contrasts were used to identify groups which differed from each other. When a linear contrast between two groups was significant ($p < 0.05$), 1-way analysis of variance (ANOVA) was used to determine the earliest divergence of the groups.

Formal pharmacokinetic analysis for the T-2 treated animals was not done because classical pharmacokinetic models assume that the animal's physiology is at steady-state, which is not true of animals undergoing acute toxicosis. However, inferences concerning pharmacokinetic changes can be drawn from the plasma concentration curves for these animals in comparison to the controls.

Results

Mean plasma concentrations versus time after IM and IV administration of dexamethasone are shown in Figures I.43 and I.44. Tables I.40 and I.41 summarize the data for the T-2 treated animals and controls. Experimental replicates for treatment groups were not statistically different (ANCOVA), so groups given the same treatment were pooled.

Plasma concentrations of dexamethasone were not significantly different between the groups given the drug IV immediately or 1 h after administration of T-2 toxin (Fig. I.43). However, plasma concentrations from both T-2 toxin dosed groups differed significantly from controls from 2.5 h on ($p < 0.05$). In contrast, plasma dexamethasone concentrations from animals given the drug IM immediately after the toxin did not differ overall from controls for at least 8 h, after which they diverged significantly (Fig. I.44). Plasma concentrations of dexamethasone in animals given the drug IM following a 1 h treatment delay were significantly higher than those treated immediately or the controls by 1 and 4 h, respectively.

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[i]Series-10, Perkin-Elmer, Norwalk, CT.

[j]Model 3551-40, Hitachi, Japan.

[k]Model 3390, Hewlett Packard, Avondale, PA.

[l]Model LC-85, Perkin-Elmer, Norwalk, CT.

[m]Whatman, Inc., Clifton, NJ.

Discussion

Previous work had shown that the route of administration had no discernible effect on the distribution and elimination of dexamethasone from the body of normal rats (Wong-Pack, 1987). It was expected that treatment with dexamethasone immediately after exposure to T-2 toxin might help maintain a normal pharmacokinetic and disposition profile of plasma dexamethasone. ANCOVA followed by 1-way ANOVA at each time point showed that when given IV immediately or 1 h after T-2, the disposition of dexamethasone was unaffected for at least 2.5 h. After that time, plasma dexamethasone concentrations following IV injection, regardless of time of administration, were similar and significantly higher than the controls (linear contrasts). However, animals given dexamethasone IM immediately after T-2 toxin had plasma drug concentrations which were statistically indistinguishable for up to 8 h from normal rats also dosed IM, after which there was a divergence. Similarly, plasma dexamethasone concentrations in animals treated IM 1 h after the toxin were not statistically different from controls for at least 2.5 h.

The percentage differences from the controls, after correction for the time of administration, are depicted in Fig. I.45. These differences can be explained in part by changes in the cardiovascular and peripheral circulatory systems. Sirén and Feuerstein (1986) found that blood flow in the hind quarter of Sprague-Dawley rats was significantly impaired within 1 h after giving T-2 toxin (1 mg/kg, IV). Six h after toxin administration, blood flow in the hind quarter had decreased 77 ± 9% (mean ± SE) and peripheral vascular resistance had increased 323 ± 69%. In addition, there was a significant decrease (about 20%) in perfusion of the renal vascular bed after 1 h; the largest decline (76 ± 13%) was achieved 4 h after T-2 toxin injection. Similarly, renal blood flow in pigs declined 27 and 82% by 1.5 and 6 h after administration of T-2 toxin (2.4 mg/kg, IV) (Lundeen et al., 1986).

The physiologic changes of decreased blood flow and increased vascular resistance are consistent with our observations that elimination of dexamethasone in animals given the drug 1 h after T-2 toxin was compromised by 2.5 h. In the absence of a profound therapeutic effect, the physiologic changes would also explain the decreased elimination of dexamethasone in rats given the drug IV immediately following the toxin. However, animals that received immediate IM administration of dexamethasone had plasma drug concentrations which were similar to IM dosed control animals for 8 h. The reason for the relatively uncompromised elimination of the drug is not evident and does not appear to be related to gross or systematic errors in our protocol. The results were reproducible.

Drug disposition under acute physiological stress is unpredictable. For example, in dogs with acute hyperkalemia, blood concentrations of digoxin were consistently and significantly higher than normal animals after 15 min (Marcus et al., 1969). In contrast, Lloyd and Taylor (1974) showed that in comparison to controls, digoxin concentrations were significantly increased after 5 min but significantly decreased after 1 h in animals experiencing hemorrhagic shock.

Effects on elimination rates of dexamethasone were dependent upon the route and timing of its injection in relation to T-2 toxin administration. Our study points out the importance of monitoring plasma

drug concentrations in acute situations because they cannot be consistently predicted.

Acknowledgements

The authors thank M. Busse, T. Keferlis, B. Kindler, R. McCartney, R. Manuel, Drs. R. Lovell, and V. Pang for their technical assistance. Supported in part by the U.S. Army Medical Research and Acquisition Activity: Contract No. DAMD17-85-C-5224. The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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Table I.40 Dexamethasone plasma concentrations (μg/ml) following IV administration in rats with acute T-2 mycotoxicosis (0.75 mg/kg, IV).

		Hours post-T-2 dosing									
		0.08	0.33	0.67	1.00	2.5	4.0	6.0	8.0	10.0	14.0
Control											
Mean	(n = 22)	12.9	12.0	10.0	8.8	5.5	3.7	2.1	1.1	0.8	0.3
	S.D.	1.6	1.1	1.1	1.3	0.8	0.5	0.4	0.3	0.2	0.1
Dexamethasone IV immediately after T-2 dosing											
Mean	(n = 13)	14.1	12.7	10.5	9.2	6.3*	4.5	2.7	1.7	1.2	0.8
	S.D.	1.5	1.3	0.8	0.6	0.5	0.6	0.5	0.5	0.4	0.3
Dexamethasone IV 1 h after T-2 dosing											
Mean	(n = 13)	14.6	13.1	11.0	9.5	6.6*	4.6	2.9	2.0	1.5	0.9
	S.D.	2.9	1.5	1.4	1.2	1.0	0.9	0.8	0.6	2.7	0.4

*Denotes starting point of significant trends from control at $P \leq 0.05$.

Table I.41 Dexamethasone plasma concentrations (μg/ml) following IM administration in rats with acute T-2 mycotoxicosis (0.75 mg/kg, IV).

		Hours post-T-2 dosing									
		0.08	0.33	0.67	1.00	2.5	4.0	6.0	8.0	10.0	14.0
Control											
Mean	(n = 22)	8.5	11.5	11.0	9.8	6.7	4.2	2.4	1.4	0.8	0.3
	S.D.	3.1	2.2	1.1	1.1	0.9	0.5	0.4	0.4	0.3	0.2
Dexamethasone IM immediately after T-2 dosing											
Mean	(n = 10)	7.8	11.1	10.7	9.8	6.6	4.6	2.6	1.7	1.2*	0.7
	S.D.	1.9	1.1	0.3	0.5	0.4	0.4	0.3	0.3	0.3	0.1
Dexamethasone IM 1 h after T-2 dosing											
Mean	(n = 10)	9.7	11.6	12.2	10.3	7.1	5.2*	3.4	2.1	1.7	1.2
	S.D.	3.2	2.0	1.2	1.0	1.0	1.1	0.8	0.5	0.2	0.3

*Denotes starting point of significant trends from control at $P \leq 0.05$.

Figure I.43 Semilogarithmic plot of averaged dexamethasone plasma concentrations in rats following IV administration of 10 mg/kg dexamethasone alone (■, n = 22) and immediately (o, n = 13) or 1 h after (Δ, n = 13) giving T-2 toxin (0.75 mg/kg, IV). Plasma concentrations of dexamethasone differed significantly from the controls for the latter two groups beginning at 2.5 h (arrow).

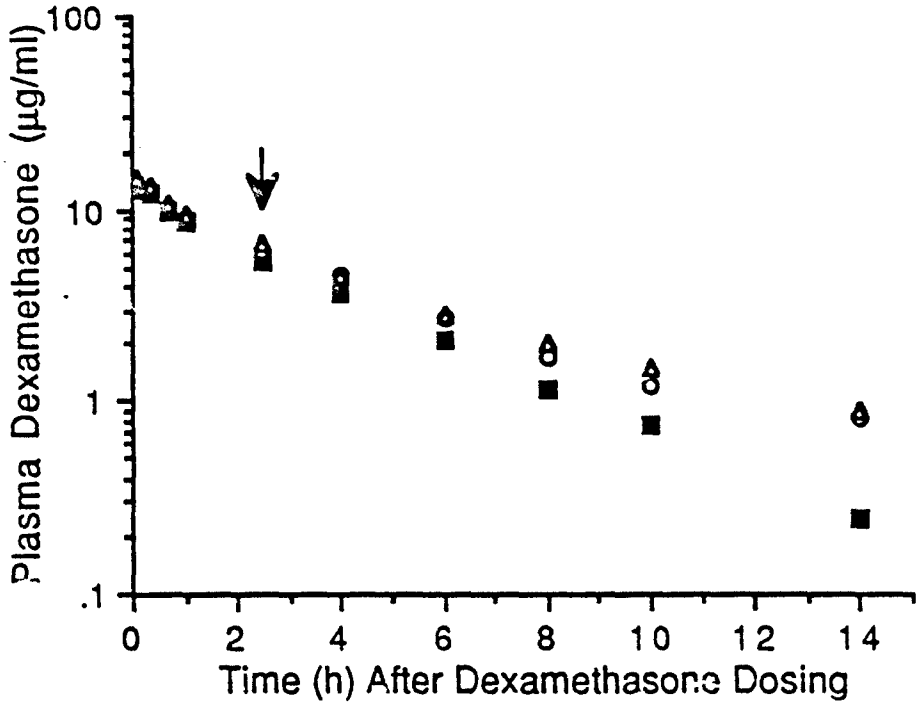


Figure I.44 Semilogarithmic plot of averaged dexamethasone plasma concentration in rats following IM administration of 10 mg/kg dexamethasone only (■, n = 17) and immediately (o, n = 10) or 1 h after (Δ, n = 10) giving T-2 toxin (0.75 mg/kg, IV). Plasma concentrations of dexamethasone for the immediate and delayed therapy groups differed from the controls by 10 and 4 h, respectively (arrows), but not from each other.

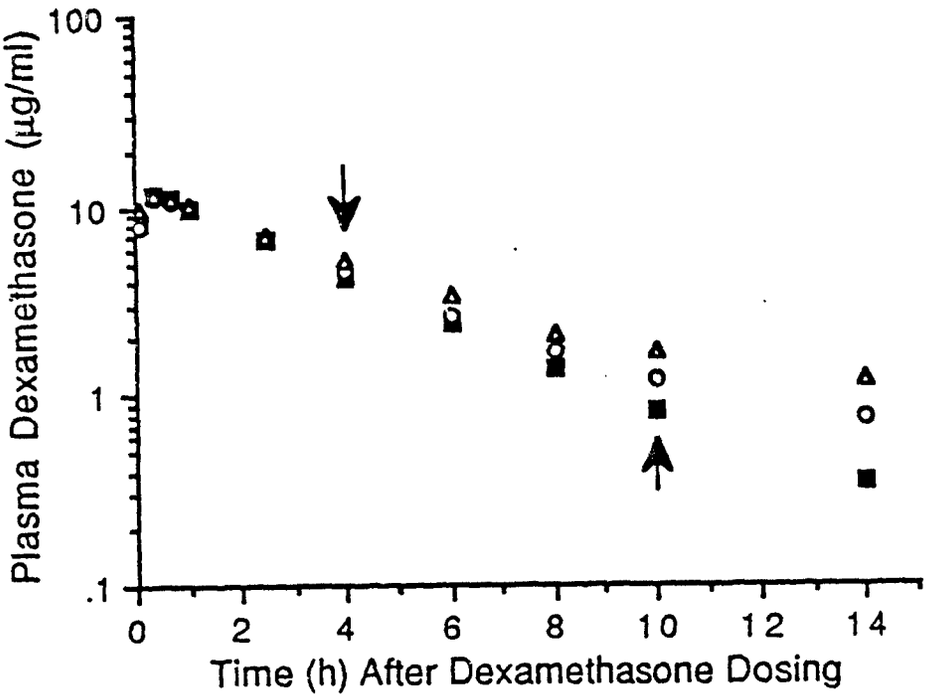
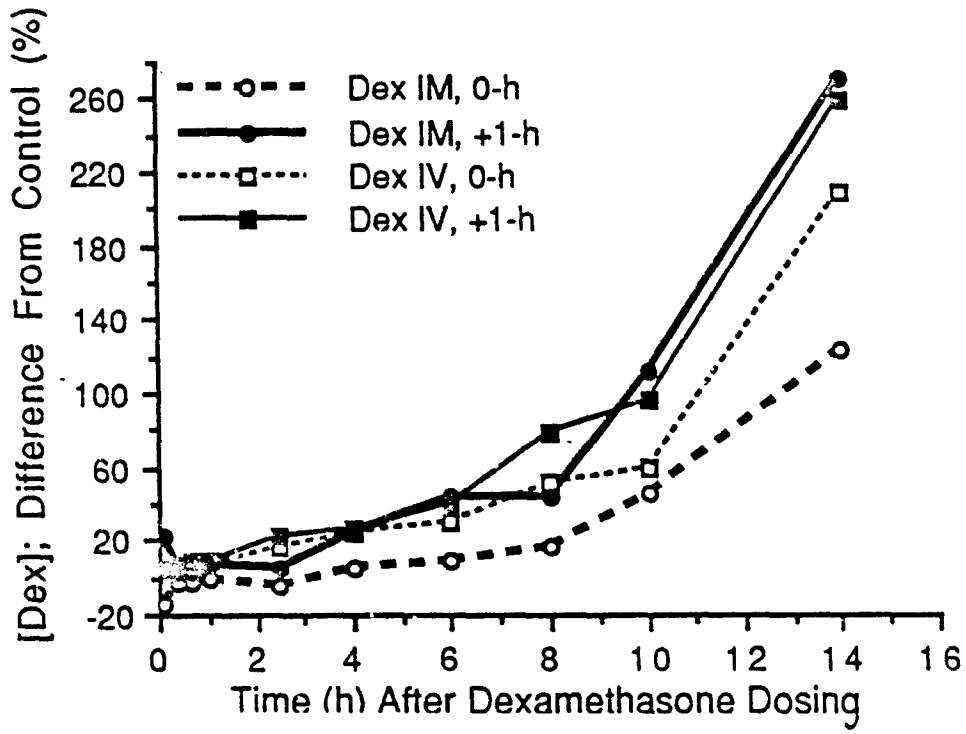


Figure I.45 Percentage change in average dexamethasone plasma concentrations from the control, after correction for the time of administration. Dexamethasone 10 mg/kg was administered IV, immediately (□, n = 13) or 1 h after (□, n = 13) giving T-2 toxin (0.75 mg/kg, IV). Alternatively, dexamethasone 10 mg/kg was administered IM, immediately (o, n = 10) or 1 h after (●, n = 10) injection of T-2 toxin.



3. Effect of repeated intramuscular administration of dexamethasone sodium phosphate on the survival of rats with acute t-2 toxicosis

by
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W. B. Buck, B. L. Kindler, and D. J. Schaeffer

Summary

Dexamethasone (10 mg/kg) was given intramuscularly (IM) as a single dose, or in a multiple dosage regimen, beginning 1 h after intravenous (IV) T-2 toxin administration (1 mg/kg). Subsequent doses (from 1 to 7) of dexamethasone and/or saline were given at 5 h intervals thereafter. Multiple dosing had no beneficial effect. Neither median survival time nor mortality differed significantly from the T-2 toxin dosed controls. However, the median survival time decreased as the number of doses of dexamethasone increased.

Introduction

T-2 toxin, a trichothecene mycotoxin, is a secondary metabolite produced by several species of Fusarium and is associated with mycotoxicosis in farm animals and man (Ueno, 1984; Joffe, 1986). T-2 toxin gained international attention after it was implicated as a component of "Yellow Rain," a chemical warfare agent allegedly used in Southeast Asia and Afghanistan (Haig, 1982).

Acute systemic T-2 toxicosis results in cardiovascular failure which is similar in character to circulatory shock (Feuerstein et al., 1985; Lorenzana et al., 1985). Due to similarities in their pathophysiology, therapeutic regimens for T-2 toxicosis have been based, in part, on those used in treating shock. Glucocorticoids, such as dexamethasone, have been recommended as a component of shock therapy (Dietzman et al., 1969; Haskins, 1983).

Though the mechanism is not known, administration of dexamethasone increased survival times and in some cases decreased lethality of T-2 toxin in mice (Ryu et al., 1987), rats (Tremel et al., 1985; Shohami et al., 1987), and pigs (Poppenga et al., 1987). As opposed to a single dose, multiple doses would result in higher plasma and tissue concentrations of dexamethasone during the extended initial acute phase (24 h) of T-2 mycotoxicosis. Four doses given to mice including 3 before the toxin (Ryu et al., 1987), 3 doses given to rats at about 12 h intervals, including 1 pre-toxin (Tremel et al., 1985), and 3 doses given to rats at about 24 h intervals (Shohami et al., 1987), all improved survival time or rates of the respective species. This study was conducted to determine whether repeated doses of dexamethasone, given IM at intervals of twice the plasma half-life, were more effective than a single treatment in prolonging the survival of rats given a lethal dose of T-2 toxin. Toxin was given IV in order to eliminate variability in the dose absorbed. Therapy was delayed for 1 h in order to obtain data reflecting potential field exposure conditions.

Materials and Methods

Female Sprague-Dawley rats (Rattus norvegicus) weighing 200 to 225 g were housed individually with free access to feed (Lab-blox, Wayne, Chicago,

IL) and water. They were housed under standard laboratory conditions (22 to 24°C and 12 h light cycle) for 10 d before experimentation. T-2 toxin (> 95% pure, produced in our laboratory) was dissolved in a 9:1 saline (0.9%):alcohol solution and administered IV at a dose of 1 mg/kg into the tail vein. One h later, dexamethasone sodium phosphate[a] was given (10 mg/kg, IM) followed by additional doses in some groups at 5 h intervals. This interval represented approximately 2 plasma elimination half-lives (Wong-Pack, 1987). Animals were randomly assigned to groups and treated according to protocols identified in Table 1. Rats were given a total of 8 injections consisting of dexamethasone or saline at specified intervals following administration of T-2 toxin, and were observed for 96 h (Fig. 1). Animals dying before that time were treated as censored observations in the lifetable analysis (Lee, 1980). The median survival time was determined using the Kaplan-Meier (1958) method. A K-sample test for censored data was employed to detect any differences in survival time among the groups (Lee, 1980). All significance tests were made at $\alpha = 0.05$.

Results

All animals in the nontoxin-treated dexamethasone control group (1) survived. Only 1 animal in the T-2 toxin positive control group (2) which did not receive dexamethasone survived until the end of the experiment. No animals receiving T-2 toxin and dexamethasone survived 96 h (Fig. I.46). Animals in treatment groups 6 and 7 generally did not survive long enough to receive more than 5 doses of dexamethasone. Because of the similar and extensive censoring due to deaths, these groups were combined for analysis. The median survival times for each of the groups are presented in Table I.42.

Discussion

The data indicate that in rats, intramuscular administration of repeated doses of dexamethasone starting 1 h after receiving a lethal IV dose of T-2 toxin did not significantly improve survival rates or times. Fricke (1985) reported that 1 dose of dexamethasone (13 mg/kg) given subcutaneously to mice, 1 h prior to or up to 3 h post-T-2 toxin administration, significantly increased the mean time to death in comparison to toxin treated positive controls.

Tremel et al. (1985) showed that rats given a single IV dose of dexamethasone (1.6 mg/kg) either 30 min prior to or 1 h after IV dosing with 0.75 mg/kg of T-2 toxin (approximate LD_{66}) had a 4-fold increase in survival rate. Delaying dexamethasone treatment 3 h was not as effective as prophylaxis or waiting only 1 h. They also found that rats given the same dose of dexamethasone at 1 h before and 12 and 24 h after an approximate LD_{99} of T-2 toxin (1 mg/kg, IV) survived longer (median 19 h; range 12 to 35 h) than T-2 toxin controls (median 9 h; range 6 to 12 h). Survival rate, however, was not improved. Shohami et al. (1987) demonstrated that dexamethasone doses as low as 1 mg/kg improved survival rates of rats given a highly lethal dose of T-2 toxin. Delaying therapy as long as 3 h resulted in increased survival times, but not rates, if the dose of dexamethasone was increased to 10 mg/kg.

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[a]Decadron®, 24 mg/ml, Merck, Sharp and Dohme, West Point, PA.

Pigs given a highly lethal dose of T-2 toxin (3.6 mg/kg, IV) had a 2-fold increase in survival time over non-treated control animals when given a therapeutic regimen of IV saline, oral superactivated charcoal, and repeated IV doses of dexamethasone (Poppenga et al., 1987). However, the relative contributions of the different components of therapy, including dexamethasone, were not defined.

Tremel et al. (1985) and Shohami et al. (1987) reported that dexamethasone administered IV to rats at 12 h or longer intervals only delayed death. Ryu et al. (1987) found that giving dexamethasone at 24, 18 and 1 h before toxin and 6 h after increased the survival rates of mice from 0 to 90%. In our study, multiple treatments with dexamethasone did not appear to increase survival time (Fig. I.46). The median survival times of the groups given repeated IM doses of dexamethasone were not significantly different from the positive controls ($p = 0.65$), although the inverse linear trend with dose approached significance ($p = 0.089$).

Our experiment showed that multiple doses of dexamethasone given after T-2 toxin offered no protection from T-2 toxicosis. Together, these studies imply that beneficial effects are due to the initial dose of the drug and that additional treatments at short intervals may in fact be detrimental.

Based on other experiments and response surface modeling (Section I.L), it appears that the effectiveness of dexamethasone is related in complex ways to its dose, the time of administration and the dose of T-2 toxin. Generally, higher doses (10 mg/kg) of dexamethasone are more effective than lower doses (2 mg/kg). Pretreatment with dexamethasone is effective when the T-2 dose is below the LD_{100}, whereas postdosing is effective when T-2 dose $\geq$ LD_{100}. The results reported here are generally consistent with this.

Acknowledgements

The authors thank T. Keferlis and R. Manuel, Drs. R. Lovell and V. Pang for their technical assistance.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation. This work was supported in part by the U.S. Army Medical Research and Acquisition Activity, Contract No. DAMD 17-85-C-5224.

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Table I.42 Experimental protocol for assessing the effect of multiple IM dosing with dexamethasone on the survival of rats with acute T-2 toxicosis and the resulting median survival times and ranges.

Group*	Treatment Number of Injections				Survival Time (hours)	
	T-2	Drug†	Saline	N‡	Median§	Range
1	0	8	0	10	96	--
2	1	0	8	6	28.2	16.0 to 96.0
3	1	1	7	12	28.7	19.6 to 53.0
4	1	2	6	12	27.0	18.1 to 40.4
5	1	4	4	12	26.6	20.9 to 42.6
6	1	6	2	12	25.3	17.9 to 42.0
7	1	8	0	12		

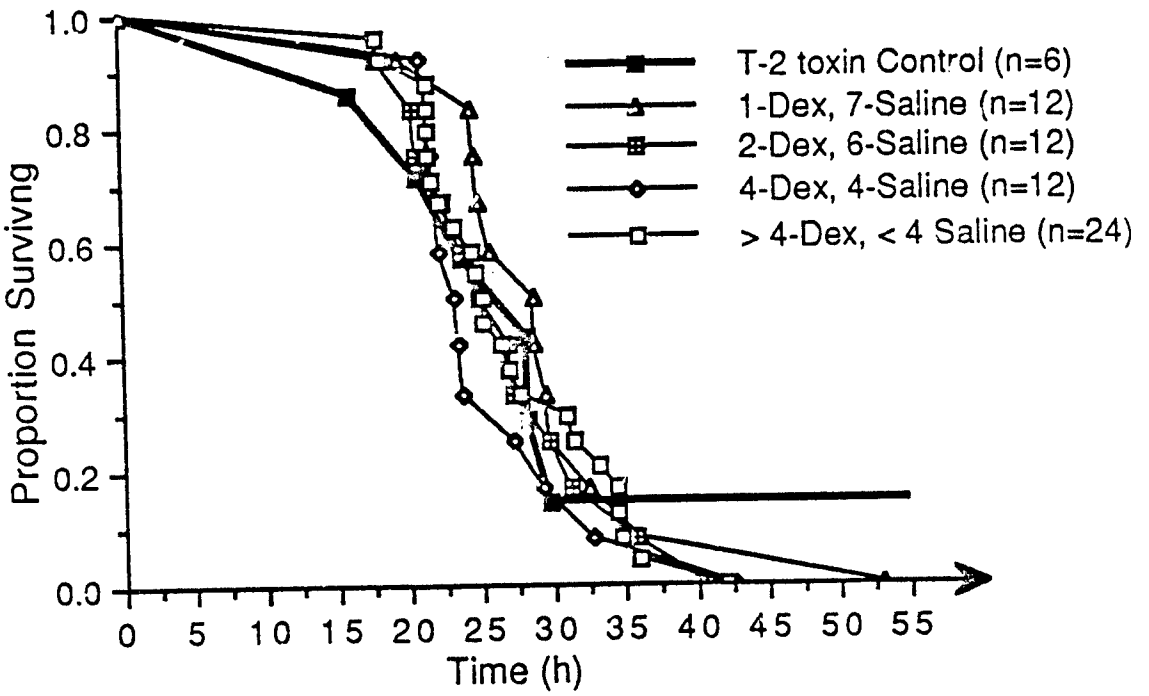
*Group 1 is the dexamethasone control. Group 2 is the T-2 toxin positive control group. Groups 3 through 7 received the indicated number of doses of dexamethasone and saline at 5 h intervals.

†Initial dose (10 mg/kg, IM) of dexamethasone was given 1 h after T-2 toxin (1 mg/kg, IV) administration. Subsequent doses of dexamethasone were given at 5 h intervals.

‡Number of animals used in the treatment group.

§Kaplan-Meier estimate of median survival time.

Figure I.46 The effect of multiple intramuscular administration of dexamethasone (dex) to rats at 10 mg/kg, 1 h after intravenous administration of T-2 toxin at 1 mg/kg. The data is presented as proportion surviving versus time for each group. Group 1 (□, n = 6), T-2 toxin positive control; Group 3 (Δ, n = 12), 1 dex and 7 saline injections; Group 4 (□, n = 12), 2 dex and 6 saline injections; Group 5 (◇, n = 12), 4 dex and 4 saline injections; Groups 6 and 7 combined (□, n = 24) greater than 4 dex injections. Animals were monitored for 96 h.



L. Evaluation of Combinations of Effective Therapeutic Agents in the Treatment of Acute T-2 Toxicosis

1. The efficacy of dexamethasone sodium phosphate and superactivated charcoal in treating rats exposed to lethal intravenous doses of T-2 toxin

by
Richard J. Lambert, William B. Buck, Barbara L. Kindler
and David J. Schaeffer

Summary

Experiments were conducted to define the optimum times and doses of dexamethasone (DEX) im and the optimum times of administration of oral superactivated charcoal (SAC, 1 g/kg) for rats given a lethal iv dose of T-2 toxin. Statistical analysis of the survival data using response surface analysis and stepwise regression yielded quadratic polynomial models which indicated the following: 1) predosing with DEX was more effective at increasing survival time than was giving DEX immediately or post T-2 exposure; 2) predosing with SAC increased survival time; 3) increasing the time between SAC doses decreased survival time; 4) when the first dose of SAC was given after the DEX, survival was decreased; and 5) when given in the proper order, the combination of DEX and SAC was of greater benefit than either treatment alone. Treatment combinations were identified which resulted in corrected survival times of 155 hr after an iv dose of toxin (1.2 mg/kg) which gave median survival times of 13 hr in sham-treatmed positive controls.

Introduction

T-2 toxin, a trichothecene mycotoxin [4,15-diacetoxy-8a-(3-methylbutyryloxy)-12,13-epoxytricho-tec-9-en-3a-ol], is a secondary metabolite which can be produced by several species of fungi in high moisture grains which are improperly stored or left in the field during the winter (Joffe, 1983). Domestic animals have been poisoned after consuming contaminated grains, and the toxin has been implicated as a component of "Yellow Rain," a chemical warfare agent allegedly used by the Soviet Union in Southeast Asia and Afghanistan (Haig, 1982).

Administration of T-2 toxin by intravenous (iv), intraperitoneal, or inhalation routes results in profound effects on the cardiovascular and lymphatic systems and a shock-like circulatory failure in a variety of species including mice, rats, guinea pigs, and swine (Feuerstein et al., 1985; Parker, 1984; Parker et al., 1984; Lorenzana et al., 1985; Lundeen et al., 1986).

High dose short-term use of corticosteroids has long been a component of therapy for circulatory shock, and the benefits include positive ionotropic effects and increased blood pressure (Dietzman et al., 1969; Haskins, 1983). The exact mechanism of their protective effects are poorly understood, but stabilization of cellular lysosomal and plasma membranes is thought to be a major benefit (Lefer, 1976; Yoffe, 1981; Fricke, 1985), in part due to phospholipase A2 inhibition (Hirata et al., 1980). Treatment with dexamethasone sodium phosphate (DEX) has been shown to increase the survival rate of mice (Fricke, 1985), rats (Tremel et al., 1985; Poppenga et al., 1988; Shohami et al., 1987; Ryu et al., 1987), and swine (Poppenga et al., 1987) given T-2 toxin. The range of

effective doses, the optimum time of administration and the benefits of multidose therapy have not been systematically evaluated.

Dietary components such as lignin fiber or alfalfa (Carson and Smith, 1983a) or adding the binding agent bentonite (Carson and Smith, 1983b) improved weight gain and feed consumption of animals exposed to T-2 toxin in the diet. The adsorbent superactivated charcoal (SAC) improved the survival rate of mice given 7 g/kg of that agent orally after receiving 5 mg/kg of T-2 toxin by the same route (Fricke and Poppenga, 1988). Treatment with superactivated charcoal has also been shown to increase the LD_{50} values of T-2 toxin given subcutaneously to mice (Fricke and Poppenga, 1988), and the survival rates and times of rats given the toxin orally (Buck and Bratich, 1986; Galey et al., 1986) or iv (Poppenga et al., 1988).

Experiments designed to define the optimum times and doses of DEX and the optimum times of administration of SAC for rats given a lethal iv dose of T-2 toxin are reported here.

Methods

Animals

Virus-free female Sprague-Dawley rats (170 to 310 g; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were housed under standard laboratory conditions (22 to 24°C and 12-hr light/dark cycle) for at least 5 days and had free access to water and laboratory chow (Lab-blox, Wayne, Chicago, IL) prior to use. Food was removed at least 12 hr and water 1 hr before dosing. Both were returned to the animals within 1 hr after receiving the toxin. Animals were numbered using a random number table, sequentially assigned to treatments, and monitored for 7 days after dosing.

Dosing Materials and Techniques

T-2 toxin produced in our laboratory from extracts of *Fusarium sporotrichioides* grown on rice cultures was > 95% pure as determined by gas chromatography with flame ionization detection of the trimethylsilylether derivative. The toxin was dissolved in 100% ethanol and diluted with 50% normal saline prior to being injected into a tail vein catheter. Dexamethasone sodium phosphate, 9-fluoro-11,17-dehydroxy-16a-methyl-21-(phosphonooxy)pregna-1,4-diene-3,20-dione disodium salt (Decadron, 24 mg/ml), was obtained from Merck, Sharp, and Dohme (West Point, PA). Each 24 mg of dexamethasone sodium phosphate was equivalent to 19.98 mg of dexamethasone alcohol. Doses which were given intramuscularly (im) are reported as the dexamethasone alcohol. Superactivated charcoal (SuperChar-Vet, Gulf Bio-Systems, Inc., Dallas, TX), which is a semi-moist powder (Lot No. GP002VA, 70% water), was mixed with deionized water to form a slurry and administered orally via a polyvinyl chloride gavage tube.

Experimental Protocols

The doses and times of administration of DEX and times of SAC or distilled deionized water (for positive controls) therapy are indicated in Table I.45a. Experiments were performed sequentially, with subsequent doses and times of administration based on the results of earlier

phases. A dose of 0.85 mg T-2 toxin per kg body weight (bwt) was given iv in Phase I, because previous studies had indicated that it was an approximate iv LD_{80} for the breed and size of rat used (Poppenga et al., 1987). The time of T-2 toxin administration was considered time "0." SAC was given at 1 g/kg bodyweight (bw) because that dose was found to be effective in prolonging survival of rats dosed orally (Galey et al., 1986) or iv (Poppenga et al., 1988) with T-2 toxin.

In Phase II, a higher dose of T-2 toxin (1 mg/kg) was given because there were a large number of survivors in Phase I. The interval between SAC doses was increased, and some groups of rats were given only one dose of that agent. Intermediate doses of DEX were given at time points indicated in Table I.45a.

There were large numbers of survivors in Phase II, so the dose of toxin was again increased, this time to 1.2 mg/kg (Phase III). Treatment protocols are given in Table I.45a.

The primary intent of these studies was to assess the response surface generated by the interactions of treatment variables. Since positive controls could not be a direct component of the response surface, they were not considered to be an integral part of the design. Some positive control animals were used in the first phase to confirm the potency of the toxin and the expected response. They were used in the last phase for the same reasons since there were an unexpected number of survivors in Phase II.

Statistical Analyses

Response surface analysis of data from preliminary single and multiple dose studies suggested that the survival time response surface defined by the SAC administration time and frequency and dexamethasone administration time and dose was complex. Response gradients appeared to be shallow and possibly saddle-shaped. For these reasons, response surface analysis was used to design sequential trials to define the shape of the response surface and the relationships among the treatment variables and survival time. As described above, an unplanned complication was the need to include T-2 dose as an independent variable in the final analysis. Response surface analysis was carried out using ECHIP version 3.2 (Wheeler, 1987). Other statistical analyses used SYSTAT version 3 (Wilkinson, 1987).

The experimental cycle for response surface analysis recommended by Wheeler (1987) is: 1) decide on resolution, 2) choose variables, 3) design experiment, 4) run experiment, 5) choose an appropriate statistical model and analyze data, 6) study the contour plot, and 7) finalize results or repeat cycle starting at 1. In these experiments, our target resolution for distinguishing differences in survival time was 6 hr. A given trial examined the control variables: dose (2, 4, 8, or 10 mg/kg) and time of DEX administration (-2, -1, 0, +1 or +2 hr), number of doses of SAC (1 or 2), the predose (-8, -6, -4, -3, or -1 hr) and postdose delay (0, +1, +2, or +4 hr) in administration of SAC and interactions between those variables.

The response surface for each trial and for the pooled data from successive trials was modeled using a quadratic polynomial. In this

model, the sum of exponents for any term is at most 2 and for at least 1 term it is 2. For example, for 2 control variables x and z, the model is:

$$\text{Survival time} = a_0+a_1x+a_{11}x^2+a_2z+a_{22}z^2+a_{12}xz$$

To account for censoring of survival times due to survivors, survival time was scaled by 168, the duration of the experiments (Finney, 1978).

Other response surface models, including linear and cubic, were also examined. Linear models, although statistically significant, gave biologically unrealistic predictions, such as increasing survival time as the post-T-2 dose delay in giving SAC increased to infinity. Most cubic models could not be fit at all or gave response surfaces which varied erratically or which showed complex and implausible reversals in response. Furthermore, since analysis of variance showed that mean scaled survival times (0.761, 0.599, 0.538) differed across T-2 doses (0.8, 1.0, 1.2 mg/kg, respectively), it was an important variable in the final model.

Results

The complex nature of the response surface was demonstrated by developing (using stepwise regression, response surface analysis, empirical model specification) many regression models having substantially different terms but with comparable r values. However, most of these models were inappropriate because they caused biologically unacceptable reversals in the response surface. For example, the model (Eq. 1) with highest predictability (r = 0.865) produced estimated survival values which increased with T-2 dose.

Eq. 1: Survival/168 = 0.435·T-2 DOSE - 0.0158·DELAY·DEXTIME - 0.00372·DELAY·DEXDOSE + 0.0215·DEXTIME·TIMEDIF - 0.139·T-2·DEXTIME

Animals in Phase II were either naive or 3-week survivors from previous experiments. Naive animals and survivors were placed in the T-2 toxin treated positive control groups and therapy group 1. Therapy groups 2, 3, and 4 consisted of all naive rats and groups 5 and 6 were all survivors. Analysis of variance showed that responses of naive animals and 3-week survivors did not differ within the control or therapy groups. Therefore, data from both types of animals were pooled in subsequent analyses. Means for the various groups are presented in Table I.43.

A 1-way analysis of variance (ANOVA) was used to examine differences among treatments in Phase II. Post-hoc analyses using linear contrasts showed that: 1) groups 3 and 4 did not differ from combined positive controls (groups 7 and 8); 2) group 5 differed from controls; 3) group 5 differed from groups 3 and 4; 4) group 5 differed from groups 3, 4, and controls; 5) groups 1, 2, and 6 did not differ from each other; 6) groups 1 and 5 differed from each other.

Subsequent analyses of data from dosing at 1.2 mg/kg T-2 used pooled data from Phase III. Stepwise regression was used to determine the relationship between survival and the independent variables DELAY (time first dose of SAC given - time DEX given); DEXTIME (time DEX given); DEXDOSE (mg/kg bwt DEX); TIMEDIF (hours between first and second dose of SAC). The model obtained for the treated groups is given by Eq. 2 (r = 0.676):

Eq. 2: Survival/168 = 0.474 - 0.145·DEXTIME - 0.00778·DEXDOSE·DELAY - 0.00744·DEXDOSE·TIMEDIF

This model indicates that: 1) predosing with DEX was more effective at increasing survival time than was giving DEX immediately or post T-2 exposure; 2) predosing with SAC increased survival time; 3) increasing the time between SAC doses decreased survival time; 4) when the first dose of SAC was given after the DEX, survival was decreased; and 5) when given in the proper order, the combination of DEX and SAC was of greater benefit than either treatment alone.

Experiments at Different Doses of T-2 Toxin

A higher dose of T-2 toxin (1.2 mg/kg) was used in the last phase because survival was higher than expected (10 to 30%) in the initial experiments where doses of 0.85 and 1.0 mg/kg were given. Statistical analysis, such as analysis of variance, showed that mean survival time/168 over all the treatments (0.761, 0.599, 0.538) differed across T-2 doses (0.8, 1.0, 1.2 mg/kg, respectively). Response surface analysis showed that the response surface was very complex. The model finally developed for the treated groups is given as Eq. 3 (r = 0.514) and selected estimates from this model illustrating the relationships between TIMEDIF, DEXTIME, and DELAY are given in Table I.44. Examples of response surfaces illustrating the relationships between dose of DEX and interval between SAC doses are shown in Figures I.47 and I.48, respectively.

Eq. 3: Survival/168 = 1.302 - 0.712·T-2 DOSE - 0.117·DEXTIME - 0.00538·DEXDOSE·DELAY - 0.00402·DEXDOSE·TIMEDIF

The relationships defined from Eq. 3 are similar to those from Eq. 2, with the additional finding that survival time decreases with increasing T-2 dose.

The previous analyses did not include the positive controls since those data were incompatible with the models in Eq. 2 or 3. However, survival data from the positive controls can be included as a correction factor. The median (mean) survival times were 53.7 hr (85.7) for a T-2 dose of 0.8 mg/kg and 12.8 hr (13.6 h) for a T-2 dose of 1.2 mg/kg. Positive control data was not obtained for a T-2 dose of 1.0 mg/kg. Using the median survival time for the respective controls as the correction factor, survival time as a result of therapy (taken over all treatments) increased about 75 hr at each T-2 dose (Tables I.45a,b). The models for the 0.8 and 1.2 mg/kg doses of T-2 toxin, uncorrected and corrected by the median positive control survival time, are given in Eq. 4 (r = 0.569) and Eq. 5 (r = 0.597), respectively. The relationships between the variables are similar to those defined by Eq. 2 and 3.

Eq. 4: Survival/168 = 1.576 - 0.934·T-2 DOSE - 0.133·DEXTIME - 0.00736·DEXDOSE·DELAY - 0.00531·DEXDOSE·TIMEDIF

Eq. 5: Survival/168 = 0.770 - 0.325·T-2 DOSE - 0.133·DEXTIME - 0.00736·DEXDOSE·DELAY - 0.00530·DEXDOSE·TIMEDIF

Table I.45b ranks by survival time those treatments which could be corrected for median survival times of controls. The relationships defined by the equations are evident in the table.

Discussion

T-2 toxin is a highly lethal mycotoxin affecting multiple organs and systems. The exact mechanism of action of T-2 toxin is not known, though it has long been postulated to be an inhibitor of protein synthesis (Ueno et al., 1973; Rosenstein and Lafarge-Frayssinet, 1983). The toxin interacts with components of the cell membrane and induces hemolysis (Segal et al., 1983), and distributes in the outer half of the phospholipid bilayer of the cell (Gyongyossy-Issa et al., 1986).

T-2 toxin also causes sufficient cell injury both in vitro (Tremel and Szinicz, 1984) and in vivo (Bunner et al., 1985) to cause leakage of intracellular enzymes. A recent study indicated that T-2 toxin significantly impaired a range of membrane functions of cultured L-6 myoblasts (Bunner and Morris, 1988). The effects occured rapidly (within 10 minutes), and at concentrations which might be expected in the tissues of animals during naturally occurring toxicoses (8.6 pM).

Treatment strategies for counteracting the in vivo effects of T-2 toxin are in their infancy. Dexamethasone has provided protective and therapeutic benefit for rats against moderately lethal (Tremel et al., 1985) and highly lethal doses of T-2 toxin (Shohami et al., 1987; Ryu et al., 1987). In the latter study, DEX doses as low as 1 mg/kg body weight given 1 hr after a dose of toxin which killed all untreated animals within 24 hr increased the survival rate to over 50%. At doses of 10 mg/kg, it was effective in prolonging survival times but not in increasing survival rates, when treatment was delayed for as long as 3 hr.

The results of this study clearly indicate that treatment with DEX and SAC provides significant protection against the lethal effects of T-2 toxin. The effectiveness of pretreatment therapy is consistent with the rapidity with which toxic effects are manifest as indicated by Bunner and Morris (1988) and the short plasma half-life of the parent compound, which ranges from 5 min in dogs (Sintov et al., 1986) to 14 min in swine and 17 min in cattle (Beasley et al., 1986). It is likely that pretreatment provides the greatest benefit because DEX is given in time to stabilize membranes prior to the toxin insult and can protect against the short-lived parent compound and toxic metabolites.

There is evidence that conjugated toxin metabolites may be reabsorbed intact or be deconjugated by bacterial or intestinal enzymes and then reabsorbed (see Section I, part B.2.c). DEX could still be of benefit after toxin exposure by reducing the effects of toxic metabolites and enterohepatically recycled toxin. Since T-2 toxin can damage cell membranes, many tissues could be affected by the toxin. Higher doses of DEX would more likely be associated with complete and rapid distribution, providing widespread membrane stabilization and increased therapeutic efficacy. In addition, doses beyond those necessary for widespread membrane stabilization should provide a greater duration of effect because of the longer period of time necessary before plasma or cell concentrations would fall below a minimal effective concentration. In addition to the initial direct damage of the toxin, there is probably a sequence of secondary effects which occur. It is likely that DEX, even after toxin exposure, would also help limit the effects of those secondary inflammatory mediators (histamine, serotonin, netrophilic enzymes, etc.).

SAC has been shown to be of benefit in treating mice exposed subcutaneously (Fricke and Poppenga, 1988) and rats given the toxin by oral (Galey et al., 1987) and iv routes (Poppenga et al., 1988). T-2 toxin has a short plasma half-life (Beasley et al., 1986; Sintov et al., 1986), and significant quantities are quickly metabolized by the liver and excreted in the bile as glucuronide conjugates (Corley et al., 1986; Pace, 1986). SAC may exert its protective effect by adsorbing toxin metabolites excreted via the bile and preventing the reabsorption of intestinally deconjugated toxin (Galey et al., 1987; Poppenga et al., 1988). In order for this process to be effective, SAC must be present at sufficient concentrations in the small intestine near the bile duct. Therefore, pretreatment with SAC is probably most effective because it places sufficient quanities of the toxin at that location. However, if given too early, it will be beyond that point. If delayed, it could prevent some degree of enterohepatic recirculation, but it would not be as effective (Galey et al., 1986; Poppenga et al., 1988). This relationship is suggested by the equations which indicate that increasing the interval between SAC doses was associated with decreased survival.

The relationship between the times of administration of SAC and DEX and the dose of DEX are complex. When SAC is given after DEX, the interaction defined by DELAY (first SAC time - DEX time) is a positive number so that the portion of the equations relating those factors is negative (- constant·DEXDOSE·DELAY). Therefore, those conditions result in decreased survival estimates which are worsened by higher doses of DEX. It is possible that DEX may be secreted (or excreted) into the stomach or first part of the duodenum and SAC administered afterward is capable of binding the drug. Why an increase in the dose of DEX, under those circumstances, should be associated with decreased survival is not clear.

The terms for interactions between DEX and SAC are included in the equations because they were found to be statistically significant. When used in the appropriate sequence, thaty indicate that the combination of DEX and SAC is more beneficial than either agent alone. Certain combinations of treatment variables resulted in corrected group survival times of 155 hr after an iv dose of toxin (1.2 mg/kg) which gave median survival times of 13 hr in sham-treated positive controls.

In conclusion, this study indicates that for the times and doses used, combination therapy can be more effective in prolonging survival than either DEX or SAC alone. Survival time is a function of the doses of toxin and DEX and the time of DEX and SAC administration.

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Table I.43 Summary of linear contrast determinations of group differences (dose of T-2 = 1.0 mg/kg).

Group		Naive/Survivor	Survival (hr)	Duncan's MLT[1]
8	Control (Positive)	N	13.063	a
8	Control (Positive)	S	14.321	a
7	Control (Saline)	S	14.417	a
7	Control (Saline)	N	19.942	a
4	SAC (+2), DEX (+2, 10)	N	24.005	a
3	SAC (+2), DEX (+2, 2)	N	32.425	a
5	SAC (0), DEX (0, 10)	S	62.500	b
1	SAC (-2), DEX (-2, 2)	N	122.775	c
6	SAC (-6), DEX (-2, 10)	S	142.500	c
2	SAC (-2), DEX (-2, 10)	N	156.975	c
1	SAC (-2), DEX (-2, 2)	S	161.750	c

[1]Duncan's Multiple Range Test. Groups with the same letter are statistically similar ($p > 0.05$).

Table I.44 Survival estimated from equation 3. DEXTIME = time DEX given, TIMEDIF = hours between first and second dose of SAC, DELAY = time first dose of SAC given - time DEX given.

T-2 (mg/kg)	TIMEDIF (hr) 0	1	2	3	4	5
	DEXTIME = -2 hr DEXDOSE = 10 mg/kg					
	DELAY = -2 hr					
0.80	1.0741	1.0339	0.9937	0.9535	0.9133	0.8731
1.00	0.9318	0.8916	0.8514	0.8112	0.7710	0.7308
1.20	0.7894	0.7492	0.7090	0.6688	0.6286	0.5884
	DELAY = 0 hr					
0.80	0.9665	0.9263	0.8861	0.8459	0.8057	0.7655
1.00	0.8242	0.7840	0.7438	0.7036	0.6634	0.6232
1.20	0.6818	0.6416	0.6014	0.5612	0.5210	0.4808
	DELAY = 2 hr					
0.80	0.8589	0.8187	0.7785	0.7383	0.6981	0.6579
1.00	0.7166	0.6764	0.6362	0.5960	0.5558	0.5156
1.20	0.5742	0.5340	0.4938	0.4536	0.4134	0.3732
	DEXTIME = 0 hr DEXDOSE = 10 mg/kg					
	DELAY = -2 hr					
0.80	0.8406	0.8004	0.7602	0.7200	0.6798	0.6396
1.00	0.6982	0.6580	0.6178	0.5776	0.5374	0.4972
1.20	0.5559	0.5157	0.4755	0.4353	0.3951	0.3549
	DELAY = 0 hr					
0.80	0.7330	0.6928	0.6526	0.6124	0.5722	0.5320
1.00	0.5906	0.5504	0.5102	0.4700	0.4298	0.3896
1.20	0.4483	0.4081	0.3679	0.3277	0.2875	0.2473
	DELAY = 2 hr					
0.80	0.6254	0.5852	0.5450	0.5048	0.4646	0.4244
1.00	0.4830	0.4428	0.4026	0.3624	0.3222	0.2820
1.20	0.3407	0.3005	0.2603	0.2201	0.1799	0.1397
	DEXTIME = 2 hr DEXDOSE = 10 mg/kg					
	DELAY = -2 hr					
0.80	0.6070	0.5668	0.5266	0.4864	0.4462	0.4060
1.00	0.4647	0.4245	0.3843	0.3441	0.3039	0.2637
1.20	0.3223	0.2821	0.2419	0.2017	0.1615	0.1213
	DELAY = 0 hr					
0.80	0.4994	0.4592	0.4190	0.3788	0.3386	0.2984
1.00	0.3571	0.3169	0.2767	0.2365	0.1963	0.1561
1.20	0.2147	0.1745	0.1343	0.0941	0.0539	0.0137
	DELAY = 2 hr					
0.80	0.3918	0.3516	0.3114	0.2712	0.2310	0.1908
1.00	0.2495	0.2093	0.1691	0.1289	0.0887	0.0485
1.20	0.1071	0.0669	0.0267	0.0000	0.0000	0.0000

Table I.45a Summary of all T-2 doses and treatment combinations evaluated with mean survival times and corrected survival times.

Dose T-2		DOSE DEX	DEXTIME[1]	Time (hr)					
(mg/kg)	N	(mg/kg)	h	SAC1	SAC2	DELAY[2]	TIMEDIF[3]	SURV	SURVCOR[4]
0.85	2	2	-1	-2	0	-1	2	19.50	-34.200
0.85	2	2	-1	-2	4	-1	6	95.175	41.475
0.85	2	2	-1	0	4	1	4	168.000	114.300
0.85	2	10	-1	-2	0	-1	2	168.000	114.300
0.85	2	10	-1	-2	4	-1	6	168.000	114.300
0.85	2	10	-1	0	4	1	4	168.000	114.300
0.85	2	2	1	-2	0	-3	2	93.000	39.300
0.85	2	2	1	-2	4	-3	6	168.000	114.300
0.85	2	2	1	0	4	-1	4	38.425	-15.275
0.85	2	10	1	-2	0	-3	2	168.000	114.300
0.85	2	10	1	-2	4	-3	6	168.000	114.300
0.85	2	10	1	0	4	-1	4	111.165	57.465
1.00	2	4	-2	-6		-4	0	160.000	
1.00	1	4	-2	-6	0	-4	6	20.250	
1.00	2	4	-2	-1		1	0	126.250	
1.00	2	4	-2	-1	4	1	5	90.750	
1.00	2	4	-2	2		4	0	108.500	
1.00	4	8	-2	-6		-4	0	128.125	
1.00	2	8	-2	-1		1	0	160.000	
1.00	1	8	-2	-1	4	1	5	160.000	
1.00	2	8	-2	2		4	0	106.750	
1.00	2	4	0	-6	0	-6	6	57.250	
1.00	2	4	0	-1		-1	0	160.000	
1.00	2	4	0	-1	4	-1	5	43.750	
1.00	1	4	0	2		2	0	16.000	
1.00	2	8	0	-6		-6	0	45.675	
1.00	2	8	0	-6	0	-6	6	41.000	
1.00	1	8	0	-1		-1	0	160.000	
1.00	1	8	0	-1	4	-1	5	31.000	
1.00	2	8	0	2		2	0	51.750	
1.00	2	8	2	-6	0	-8	6	160.000	
1.20	2	2	-2	-6	0	-4	6	104.250	91.450
1.20	2	2	-2	-6	4	-4	10	107.375	94.575
1.20	18	2	-2	-2		0	0	140.097	127.297
1.20	2	2	-2	-1	4	1	5	89.500	76.700
1.20	2	4	-2	-6	0	-4	6	168.000	155.200
1.20	2	4	-2	-1	4	1	5	53.400	40.600
1.20	2	8	-2	-6	0	-4	6	142.200	129.400
1.20	2	8	-2	-1	4	1	5	32.790	19.990
1.20	6	10	-2	-6		-4	0	134.000	121.200
1.20	2	10	-2	-6	0	-4	6	90.200	77.400
1.20	2	10	-2	-6	4	-4	10	36.115	23.315
1.20	10	10	-2	-2		0	0	156.975	144.175
1.20	2	10	-2	-1	4	1	5	30.260	17.460
1.20	2	2	0	-6	0	-6	6	95.000	82.200
1.20	2	2	0	-6	4	-6	10	95.000	82.200
1.20	2	2	0	-1	4	-1	5	38.780	25.980
1.20	2	4	0	-6	0	-6	6	59.395	46.595
1.20	2	4	0	-1	4	-1	5	25.500	12.700
1.20	1	8	0	-6	0	-6	6	71.000	58.200
1.20	2	8	0	-1	4	-1	5	46.250	33.450
1.20	1	10	0	-6	0	-6	6	168.000	155.200
1.20	2	10	0	-6	4	-6	10	19.750	6.950
1.20	2	10	0	-1	4	-1	5	106.200	93.400
1.20	8	10	0	0		0	0	62.500	49.700
1.20	10	2	2	2		0	0	32.425	19.625
1.20	2	10	2	-6		-8	0	168.000	155.200
1.20	10	10	2	2		0	0	24.005	11.205

[1]DEXTIME is the time of DEX administration with the time of T-2 dosing = 0.
[2]DELAY is the time the first dose of SAC was given minus the time DEX was given.
[3]TIMEDIF is the number of hours between the first and second doses of SAC.
[4]Median survival times for positive control animals were subtracted from mean survival times to provide corrected survival times.

able I.45b Mean survival times for treated animals (corrected for median survival time of controls). Treatments are arranged in order from those associated with shortest survival times to those with the longest.

ose T-2		DOSE DEX	DEXTIME[1]	Time (hr)				
mg/kg)	N	(mg/kg)	h	SAC1	SAC2	DELAY[2]	TIMEDIF[3]	SURVIVAL-C
0.85	2	2	-1	-2	0	-1	2	-34.165
0.85	2	2	1	0	4	-1	4	-15.240
1.20	2	10	0	-6	4	-6	10	7.000
1.20	10	10	2	2		0	0	11.255
1.20	2	4	0	-1	4	-1	5	12.750
1.20	2	10	-2	-1	4	1	5	17.510
1.20	10	2	2	2		0	0	19.675
1.20	2	8	-2	-1	4	1	5	20.040
1.20	2	10	-2	-6	4	-4	10	23.365
1.20	2	2	0	-1	4	-1	5	26.030
1.20	2	8	0	-1	4	-1	5	33.500
0.85	2	2	1	-2	0	-3	2	39.335
1.20	2	4	-2	-1	4	1	5	40.650
0.85	2	2	-1	-2	4	-1	6	41.510
1.20	2	4	0	-6	0	-6	6	46.645
1.20	8	10	0	0		0	0	49.750
0.85	2	10	1	0	4	-1	4	57.500
1.20	1	8	0	-6	0	-6	6	58.250
1.20	2	2	-2	-1	4	1	5	76.750
1.20	2	10	-2	-6	0	-4	6	77.450
1.20	2	2	0	-6	0	-6	6	82.250
1.20	2	2	0	-6	4	-6	10	82.250
1.20	2	2	-2	-6	0	-4	6	91.500
1.20	2	10	0	-1	4	-1	5	93.450
1.20	2	2	-2	-6	4	-4	10	94.625
0.85	2	2	-1	0	4	1	4	114.335
0.85	2	2	1	-2	0	-3	6	114.335
0.85	2	10	-1	-2	4	-1	2	114.335
0.85	2	10	-1	0	4	-1	6	114.335
0.85	2	10	-1	-2	4	1	4	114.335
0.85	2	10	1	-2	0	-3	2	114.335
0.85	2	10	1	-2	4	-3	6	114.335
1.20	6	10	-2	-6		-4	0	121.250
1.20	18	2	-2	-2		0	0	127.347
1.20	2	8	-2	-6	0	-4	6	29.450
1.20	10	10	-2	-2		0	0	144.225
1.20	2	4	-2	-6	0	-4	6	155.250
1.20	1	10	0	-6	0	-6	6	155.250
1.20	2	10	2	-6		-8	0	155.250

DEXTIME is the time of DEX administration with the time of T-2 dosing = 0.
DELAY is the time the first dose of SAC was given minus the time DEX was given.
TIMEDIF is the number of hours between the first and second doses of SAC.

Figure I.47 Effect of the dose of DEX on the scaled survival time. Dose of T2 toxin = 1.2 mg/kg, DEXDOSE = 2.0 mg/kg (thick lines), DEXDOSE = 10.0 mg/kg (thin lines), TIMEDIF (interval between doses of superactivated charcoal) = 0 (one dose of superactivated charcoal). DELAY = time first dose of SAC given - time dexamethasone given: DEXTIME = time dexamethasone given. Under the same conditions there is increased survival at the higher dose of DEX.

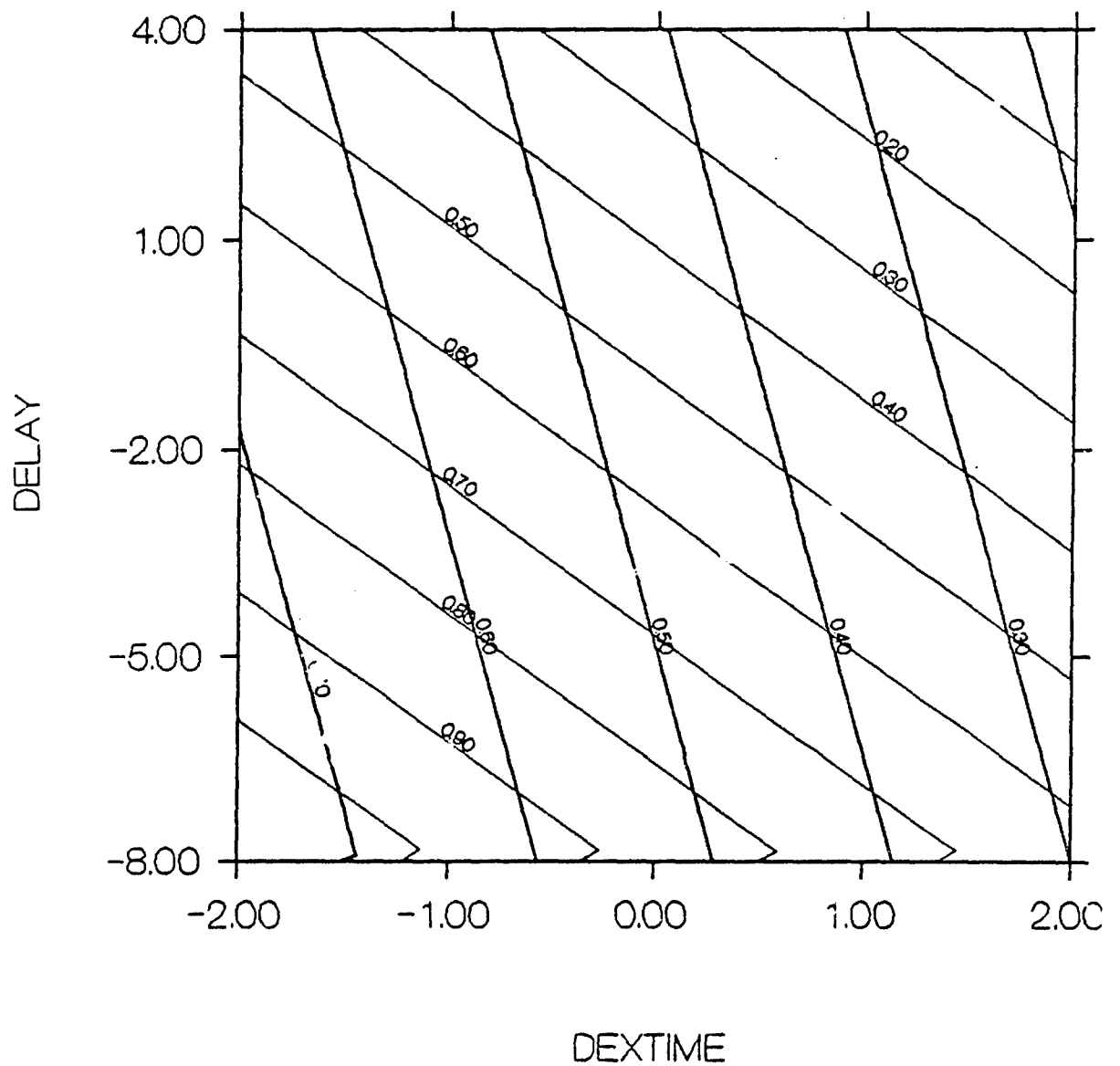
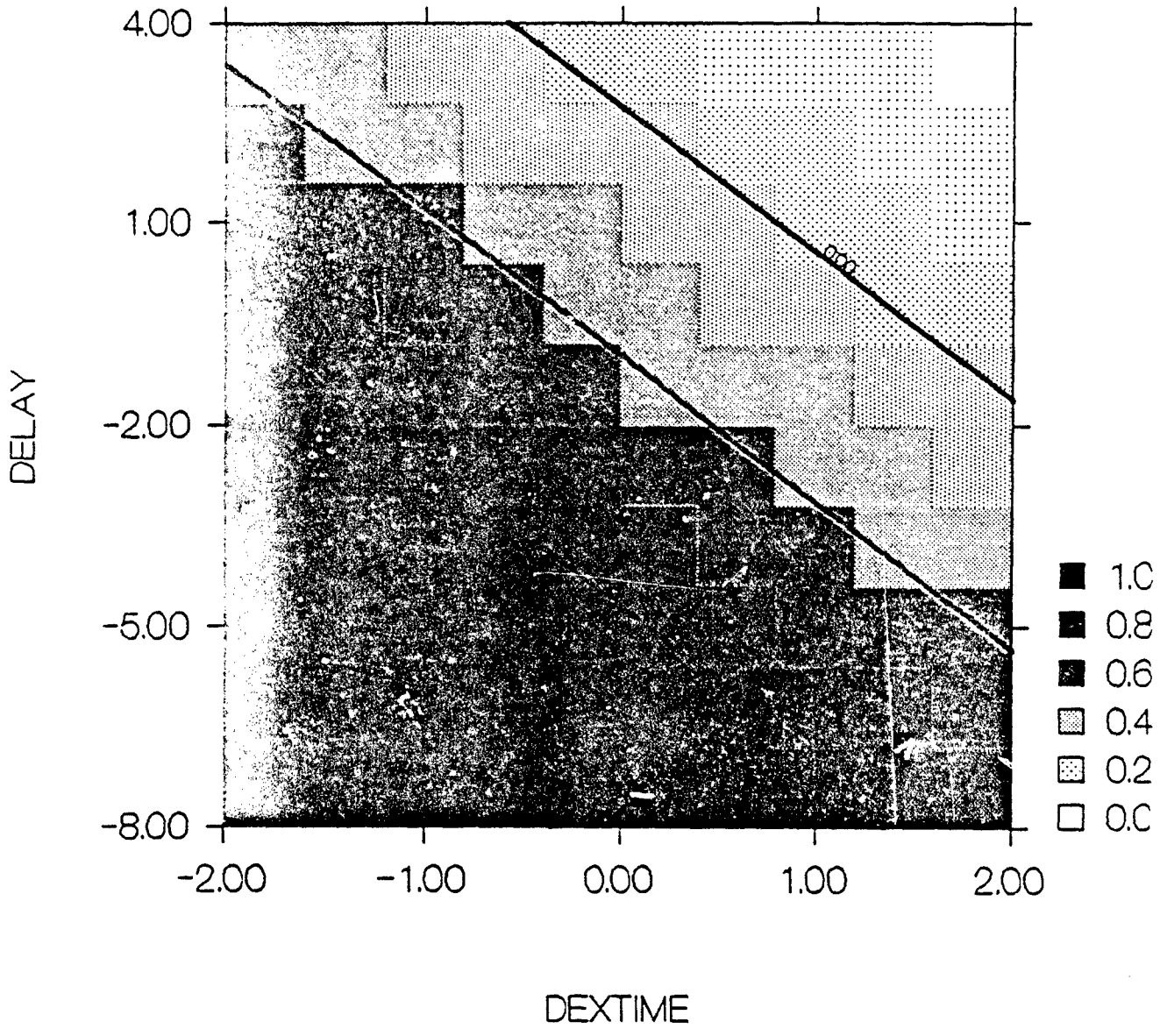


Figure I.48 Effect of increasing the interval between SAC doses (TIMEDIFF) from 2.5 hr (shaded area) to 7.5 hr apart (diagonal lines) on the scaled survival time. Dose of T-2 toxin = 1.2 mg/kg, DEXDOSE = 10.0 mg/kg, TIMEDIF = 0. Increasing the time interval between doses is associated with decreased survival.



M. Therapy Studies: General Discussion and Conclusions

by
Robert H. Poppenga, Val R. Beasley, and William B. Buck

Acute T-2 toxicosis is characterized by a complex pathophysiology involving multiple organ systems. One feature of the acute toxicosis seems to play an important role in toxin-induced mortality: the relatively rapid onset of circulatory shock as indicated by a decline in cardiac output and aortic mean pressure, the occurrence of lactic acidosis, and organ blood flow changes (Lorenzana et al., 1985a; Feuerstein et al., 1985; Lundeen et al., 1986; Siren and Feuerstein, 1986; Beasley et al., 1987). Whether the circulatory shock is a direct result of the action of the toxin, secondary to the release of vasoactive substances from cell destruction and ischemic tissues, or both has not been determined. Circulatory derangements have been hypothesized to be due to autonomic nervous system alterations, direct cardiotoxicity, and altered endothelial membrane permeability (Yarom et al., 1983; Feuerstein et al., 1985; Wilson and Gentry, 1985). The concentrations of a number of vasoactive substances have been measured in rats, guinea pigs, and swine exposed to acutely toxic doses of T-2 toxin (Lorenzana et al., 1985a; Feuerstein et al., 1985; Siren and Feuerstein, 1986). These include catecholamines such as norepinephrine and epinephrine; prostaglandins such as thromboxane A_2, prostacyclin, and leukotriene C_4; vasopressin; and renin. However, alterations in concentrations of other important vasoactive substances such as myocardial depressant factor, endogenous opioids, and monokines have not been investigated. These appear to play an important role in the pathophysiology of other circulatory shock states (Lefer, 1982; Lechner et al., 1985; Filkins, 1985).

Complicating our understanding of acute T-2 toxicosis has been the failure to identify all the important cellular mechanisms of action of the toxin. Interpretation of *in vitro* data has been difficult due to the various systems utilized and, especially, the wide range of T-2 toxin concentrations and incubation times used.

It is almost universally agreed that T-2 toxin is a potent inhibitor of eukaryotic cell protein synthesis (Ueno et al., 1973; Rosenstein and Lafarge-Frayssinet, 1983; Trusal, 1985). What is not agreed upon is the role that this cellular mechanism of action plays in the acute pathophysiology and lethality following exposure to sufficient toxin. There is some thought that protein synthesis inhibition may require a much longer time frame (24 to 48 hr) for lethality to result than that which occurs following acute exposure to T-2 toxin (Bridges et al., 1983; Shier, 1985). Unfortunately, studies in support of this conclusion appear to be lacking. It seems reasonable to assume that different cell types are more or less susceptible to the rapidity of onset of effects following protein synthesis inhibition and this may be one of the primary reasons that rapidly dividing cells in lymphoid tissues, bone marrow, and the intestinal tract are particulary sensitive.

The effect of T-2 toxin on the cell membrane may prove to be of major importance with regard to the rapidity of cell destruction. Of particular interest would be the possible presence of a cell membrane receptor for which binding of the toxin would be the first critical step in the expression of cell toxicity as has been suggested (Gyongyossy-Issa et al., 1985). Whether or not a cell membrane receptor exists, there is some evidence that T-2 toxin may interact with the cell membrane in a more general way, possibly intercalating into the membrane and causing loss of normal membrane structure

and function. DeLoach et al. (1987) has speculated that the abnormal shape of rat erythrocytes following incubation with T-2 toxin is due to the interaction of the toxin with the inner lipid bilayer of the plasma membrane and may require the presence in the membrane of phosphatidylcholine. The data of Bunner and Morris (1988) strongly suggest that a direct cell membrane effect may be the reason for the acute toxicity of T-2.

Much of the current interest in circulatory shock research has focused on cell energetics and the role that depletion of high energy phosphate stores plays in cell destruction (Chaudry, 1985). This is an area which has received relatively little attention in the effort to define the cellular mechanisms of action of T-2 toxin. There is some evidence that the toxin can cause ultrastuctural abnormalities in mitochondria (Trusal, 1985) although in vitro work attempting to elucidate the role of T-2 toxin on mitochondrial respiration have employed artificially high concentrations of the toxin (Pace and Murphy, 1981; Schiller and Yagan, 1981; Pace, 1983).

The foregoing discussion should point out two things: 1) that acute T-2 toxicosis is complex and involves many organ systems and 2) that our knowledge concerning the cellular mechanism of action(s) of the toxin is lacking despite an explosion of recent information. On the one hand, these 2 factors have made the formulation of effective therapies more difficult. On the other hand, there would seem to be almost limitless scientifically justifiable possibilities for investigation.

There are many similarities between the pathophysiology of acute T-2 toxicosis and endotoxicosis. Indeed, it is possible that endotoxin plays a role in acute T-2 toxicosis, although there is no experimental proof for this. Both toxins cause rapid onset of circulatory shock and organ blood flow alterations (Lundeen et al., 1986; Beasley et al., 1987; Ferguson et al., 1978; Fish et al., 1986; Wyler et al., 1969). Blood flow alterations reflect an attempt to maintain blood flow to vital organs, such as the brain and heart, at the expense of less critical visceral organs, such as the spleen and pancreas. The hemodynamic alterations are in large part due to changes in the concentrations of vasoactive substances such as catecholamines and prostaglandins. Initial hematologic responses are similar and probably reflect a response to stress. Common serum chemistry alterations include decreases in serum calcium and plasma glucose (Lorenzana et al., 1985b; Hinshaw, 1982; Holcroft et al., 1980). Blood-gas measurements reflect the occurrence of a metabolic acidosis with an attempt at respiratory compensation (Hinshaw et al., 1977; Lorenzana et al., 1985a). Many pathologic lesions are similar, especially those involving the gastrointestinal tract and adrenals (Pang et al., 1987; Falk et al., 1985; Itoh and Guth, 1985; Hoffmann, 1977).

There are several major differences between the two toxicoses. Acute T-2 toxicosis is characterized by severe lymphoid necrosis, whereas endotoxic shock is not (Pang et al., 1987; Hinshaw, 1982). On the other hand, disseminated intravascular coagulopathy (DIC) and adult respiratory distress syndrome (ARDS) are commonly seen in endotoxic shock but have not been shown to occur in T-2 toxin-induced snock (Hinshaw, 1982; Ayres, 1982).

While different forms of shock may initially have dissimilarities, ultimately a basic pathophysiology appears to be common to all (Gann and Amaral, 1985). Due to common features of acute T-2 toxicosis and other circulatory shock states, it was decided to investigate several agents which had shown some degree of therapeutic efficacy in hemorrhagic or endotoxic circulatory shock. It should be pointed out that there is no consensus with regard to

the most effective therapy for circulatory shock. This is in spite of intensive investigation over many years.

The original goal of our work was to utilize rats to screen a number of drugs for efficacy as assessed by survival and histologic data, to pick those that showed the most benefit, and to utilize instrumented swine to gain an understanding of how the selected agents were acting to improve survival. It was felt that, as models for exposed humans, swine could be utilized to assess several agents for which the rat might not be as appropriate due to: 1) the necessity of repeated blood sampling for tailoring the therapy, as in the case of sodium bicarbonate; 2) physiologic differences such as the failure of the rat to vomit, important for assessing antiemetic efficacy; and 3) technical difficulties associated with catheter placement and maintenance in the rat, important for monitoring blood pressures in order to optimize fluid therapy. Certain adjustments in the above goal were made during the course of the research and were dependent to a significant extent on input from Army investigators. As an example, while methylprednisolone sodium succinate appeared to be efficacious in our early studies, dexamethasone sodium phosphate was employed in the swine studies due to the feeling on the part of those investigators that the latter glucocorticosteroid was more efficacious (Fricke and Poppenga, 1987).

Another important influence on the selection of therapeutic agents was the need to identify agents which were already approved for use in humans and not in developmental stages. However, as is evident from some of the therapies selected for evaluation, this did not preclude the investigation of rather unorthodox therapies such as trichodermin and ATP + $MgCl_2$.

The following discussion will focus in turn on the 3 phases of this report: 1) rat screening studies, 2) preliminary swine studies, and 3) definitive swine studies.

1. Rat studies

A number of possible therapies were assessed in rats. The selection of dosage regimens for each drug were based on their use in other shock states or were empirically formulated.

Three agents showed efficacy for the treatment of acute T-2 toxicosis in the rat. These were 2 glucocorticosteroids, methylprednisolone sodium succinate (MPSS) and dexamethasone sodium phosphate (DEX), and a superactivated charcoal (SAC).

Glucocorticosteroids have been shown to be efficacious in other shock states, particularly endotoxic shock (Lefer and Spath, 1984; Schumer, 1982). Efficacy depends to a large extent on the form and dose of the corticosteroid used and how soon after the onset of shock it is administered (Lefer and Spath, 1984). Thus, the administation of high doses of water-soluble salts of corticosteroids given soon after the onset of shock have proven to be the most efficacious (Shatney, 1982; Schumer, 1983; Lefer and Spath, 1984). While only water-soluble forms of glucocorticoids have been assessed in the treatment of animals for circulatory shock induced by T-2 toxin, the other 2 requirements for efficacy appear to be the same for T-2 toxicosis as for endotoxic shock: high doses are required and efficacy declines if there is a delay between toxin and drug administration (Tremel et al., 1985; Fricke and Poppenga, 1987). In addition, recent work by Wong-Pack (1987) suggests that

multiple doses of glucocorticosteroids do not enhance survival over that achieved following a single dose.

Glucocorticosteroids are remarkably free from acute side effects and can therefore be used at relatively high doses for short periods of time. However, one harmful effect noted was the occurrence of lympholysis in the thymic cortex of rats given MPSS ip at 30 mg/kg. Thymic lympholysis following glucocorticosteroid therapy is well documented (Metcalf, 1966). While not quantitated in the present studies, there appeared to be an additive effect of T-2 toxin and MPSS on lymphocyte destruction. Whether the additional lymphocyte destruction due to the corticosteroid would have an adverse impact on animal survival over the long-term is not known.

The reason for the efficacy of glucocorticosteroids in hemorrhagic or endotoxic circulatory shock has been extensively investigated. It appears that glucocorticosteroids are able to stabilize cell membranes, particularly lysosomal membranes (Goldfarb and Glenn, 1983; Lefer and Spath, 1984). This in turn prevents the formation of myocardial depressant factor, lysosomal enzymes, and possibly other vasoactive substances. It has also been suggested that glucocorticosteroids are able to preserve microcirculatory perfusion (Altura and Altura, 1974).

It is certainly not clear from the present studies why glucocorticosteroids were effective in lessening mortality due to T-2 toxin. In rats given a relatively high dose of T-2 toxin iv (1.5 X LD_{50}), MPSS given ip at 30 mg/kg immediately after the toxin lessened the severity of gastric lesions. The severity of lesions in other tissues, particularly the lympholysis in tissues such as the thymus and spleen and the epithelial cell necrosis in segments of the small intestines, was not alleviated. It may be that MPSS is not able to prevent primary damage induced by T-2 toxin but that it lessens cell damage which may result from secondary effects of the toxin such as the tissue ischemia caused by impairment of the microcirculation. MPSS may also preserve the viability of cells adjacent to those cells primarily affected by the toxin. Tissues from dexamethasone-treated rats (DEX + PGE_1 study) were not examined histologically.

There is sufficient evidence for the efficacy of SAC given po for the treatment of acute T-2 toxicosis following oral exposure to the toxin (Galey et al., 1987; Coddington, 1986). In addition, there is evidence that SAC given po is effective in preventing mortality in mice exposed to T-2 toxin sc (Fricke and Poppenga, 1987). In general, the results of the SAC studies reported herein support this earlier evidence. The reason for the efficacy of SAC is probably due to the interruption of enterohepatic recirculation of toxic metabolites thereby lessening local effects on the intestinal tract epithelium and decreasing systemic exposure to the toxin. This hypothesis is supported by results from histologic evaluation of tissues from the SAC studies in which there were marginally significant reductions in the severity of lesions in the duodenum and jejunum ($p = 0.054$ and 0.052, respectively) and more highly significant reductions in the ileum and closely associated lymphoid tissues ($p = 0.005$ and 0.009, respectively). Another theory, which has not been investigated, is that SAC prevents the absorption of endotoxin through a damaged intestinal epithelial barrier.

Unlike the previous findings, in the present studies involving SAC alone pretreatment was necessary for efficacy. There is some evidence that

gastrointestinal transit time is slowed in T-2 toxicosis (Galey et al., 1987). In our work, routine post-mortem examination of rats given T-2 toxin followed by SAC showed the charcoal to be largely retained in the stomach. In addition, the charcoal tended to be dry and appeared unlikely to pass into the small intestine. Thus, the administration of charcoal after toxin administration may prevent its movement to segments of the intestine where it may have maximal benefit.

As indicated in this report, the combination of superactivated charcoal and dexamethasone shows considerable promise in treating parenterally induced acute T-2 toxicosis. Treatment combinations of those two agents were identified which resulted in rat group survival times of 155 hr after an iv dose of toxin (1.2 mg/kg) which gave median survival times of 13 hr in sham-treated positive controls. However, greatest efficacy is produced by pretreating with those agents, which lessens their usefulness unless an exposure can be anticipated. In addition, it appears that when superactivated charcoal is given after the dexamethasone, it can interfere with beneficial effect of the glucocorticoid.

A number of drug agents were evaluated and found to have no efficacy. This may have been due to several factors including: 1) the use of an inadequate dosing protocol including the use of too small a dose, not treating for a long enough period of time, or using an inappropriate route of administration; 2) the use of too large a dose of T-2 toxin which would mask a beneficial action of a drug given alone; or 3) the formulation of erroneous hypotheses with regard to whether a particular cellular event or systemic process was important in the pathophysiology of T-2 toxicosis. As an example, the administration of naloxone ip was later deemed to have been inappropriate due to recognition of its extensive first-pass metabolism by the liver (McEvoy, 1987). The hypothesized beneficial action of ascorbic acid was due to its free radical scavenging ability, although a role of free radicals in acute T-2 toxicosis is unclear. Thus, the failure of ascorbic acid to show efficacy may be due to the fact that free radicals do not play a decisive role in the pathophysiology of the acute toxicosis.

The additive toxicity of trichodermin and T-2 toxin suggests that the difference in acute toxicity between the 2 trichothecenes are not due to differences in binding affinity for a common cell receptor. It is more likely that the much higher toxicity of T-2 toxin is due to its ability to interact with the cell membrane causing membrane alterations or to penetrate the cell membrane and reach intracellular target sites more readily. The latter hypothesis is supported by the in vitro work of Ueno et al. (1973) in which the ability of various trichothecenes to inhibit protein synthesis was investigated using both cell-free and whole cell systems. These authors noted that the more water-soluble trichothecenes were as effective in inhibiting protein synthesis as the more lipid soluble ones in cell-free but not in whole cell systems.

2. Preliminary swine studies

As discussed in Appendix I.A, preliminary studies were conducted in swine primarily to work out monitoring techniques. In the process, several drug agents were evaluated in these animals including the nonspecific α-blocker, phenoxybenzamine; the catecholamine, dopamine; the ß-blocker, propanolol; and the glucocorticosteroids, DEX and MPSS. A relatively high dose of T-2 toxin was used (2.4 to 3.6 mg/kg iv) and this may have

overwhelmed the ability of an individual agent to demonstrate efficacy. Although too few swine were used to make meaningful statistical evaluations of the data, several useful observations were made.

First, there was evidence that the glucocorticosteroids were effective in prolonging survival times. This supported earlier observations made in rats.

Second, while there was no improvement in survival times in 2 pigs given phenoxybenzamine iv compared to a control animal, there did appear to be clinical improvement in peripheral circulation and a reduction in the severity of diarrhea. The use of α blockers has been proposed for treatment of other shock states, primarily to counteract the peripheral vasoconstriction and organ ischemia which occurs (Lefer and Spath, 1984). In retrospect, it may have been more appropriate to use a specific α_1-receptor blocker, such as prazosin, since α_2-blockade can augment the amount of catecholamines mobilized and released by autonomic stimuli (Adams, 1983) which, in turn, may exacerbate vasoconstriction.

On the other side of the therapeutic issue is the thought that sympathomimetic agents should be used in order to increase cardiac performance and maintain arterial blood pressure (Lefer and Spath, 1984). However, the use of potent vasoconstrictors, such as norepinephrine, may exacerbate already impaired tissue perfusion. The use of dopamine in shock has been suggested as an alternative to norepinephrine due to its peripheral vasodilatory properties in the renal and splanchnic vascular beds and its positive inotropic effect on the heart (Talley et al., 1969; Lefer and Spath, 1984). However, there did not appear to be any benefit from the administration of dopamine to 2 preliminary swine in terms of survival time (mean of 6.5 hr).

The β-blocker, propranolol, was considered due to the hypothesis that the cardiac lesions resulting from toxicosis in swine were similar to those induced by high concentrations of catecholamines (Pang et al., 1985). However, it was apparent in the few animals studied that propranolol resulted in more rapid circulatory decompensation and death and its use was therefore contraindicated at the dosage employed. The mean time to death for 2 swine given T-2 toxin iv at 2.6 mg/kg T-2 toxin + propranolol was 4 hr, whereas the mean time to death for 3 control animals in the definitive study given T-2 toxin iv at 3.6 mg/kg was 8.6 hr.

There are no known reports in the literature on shock to suggest that the endogenous release of catecholamines in response to the stress of circulatory shock causes cardiotoxicity. As a result of the preliminary swine studies using propranolol, two hypotheses concerning catecholamine action in acute T-2 toxicosis are proposed: (1) that the response of the heart to early increases in catecholamines is an important compensatory action to the primary or secondary effects of T-2 toxin and (2) that cardiac lesions noted in swine following administration of high doses of T-2 toxin are not caused by the action of catecholamines.

3. Definitive swine studies

These studies were intended to evaluate a combined therapeutic protocol for acute T-2 toxicosis and were done prior to the recently completed rat combination therapy experiments. Most of the drug agents were selected with a specific goal in mind. Glucocorticosteroids were included in all

therapeutic regimens due to the efficacy shown previously. Normal saline (0.9%) was chosen in the belief that fluid administration would maintain arterial blood pressure and secondarily improve cardiac output and tissue perfusion. Sodium bicarbonate was included to counteract the lactic acidosis and associated decline in arterial blood pH which occurs in acute T-2 toxicosis. The inclusion of superactivated charcoal and magnesium sulfate was based on the efficacy in previous trials and the belief that substantial enterohepatic recirculation of toxic metabolites occurs. Metoclopramide was employed in the anticipation that it would control the emesis caused by T-2 toxin and allow for greater retention of orally administered superactivated charcoal and magnesium sulfate. This therapeutic protocol was primarily designed to maintain homeostasis and not to counteract any specific cellular actions of T-2 toxin.

The primary goal of the combination therapy in the definitive swine study was to ameliorate the degree of circulatory shock which occurs in T-2 toxin-dosed swine. Fluid administration, base replacement, and glucocorticosteroids can improve cardiovascular function in circulatory shock either directly or indirectly. Fluids can directly improve arterial blood pressure by increasing intravascular volume. This increase in blood pressure can secondarily improve organ blood flow via maintenance of perfusion pressure. Also, an increase in venous return to the heart enhances cardiac output. It would be logical to assume that, if fluid replacement is successful in maintaining organ blood flow, the release of cardioinhibitory factors such as MDF from ischemic organs would be lessened.

In the present study, the administration of large volumes of normal saline was ineffective at maintaining arterial blood pressure and cardiac output. It may be that a large percentage of the administered saline was lost via the intestines. While not measured, the volume of watery diarrhea appeared to be substantially increased in those swine given saline. In addition, based on the degree of tissue edema noted in some swine, loss of fluid into interstial tissues may have been significant. Whether this was due to the choice of a crystalloid fluid, endothelial cell damage, or both is not clear. Certainly before the efficacy of intravascular volume expansion is abandoned, the use of plasma expanders such as dextran should be investigated.

While the administration of normal saline did not appear to significantly improve the hemodynamic status of swine given T-2 toxin, it did increase urine production over control animals. This increased urine output was likely responsible for significant overall treatment effects on serum creatinine, potassium, and phosphorus concentrations; although only phosphorus was significantly decreased in the group given all therapy vs the group given all therapy less normal saline. Another possible benefit of normal saline administration was relative hemodilution as reflected in the comparison in hematocrit and serum total protein between the positive control group given no therapy and the other treatment groups. Hemodilution may lessen blood stasis and secondarily improve tissue oxygenation (Safar, 1982).

Sodium bicarbonate therapy has been recommended for the treatment of metabolic acidosis. T-2 toxin causes metabolic acidosis in rats and swine given T-2 toxin, primarily due to increases in lactic acid concentrations (Lorenzana et al., 1985a; Feuerstein et al., 1985). Acidosis due to any etiology would be expected to adversely affect the heart and reversal of the acidosis would therefore be expected to

preserve myocardial function. There was significantly lower blood pH in the positive control group as compared to the group given all therapy. Also, the group given all therapy minus bicarbonate had lower blood pH than the other treatment groups. Thus, bicarbonate administration was somewhat effective in limiting the acidemia. The moderation in metabolic acidosis was reflected in less severe changes in PaO_2 and $PaCO_2$, thus indicating less need for respiratory compensation. Nevertheless, the amelioration of the decline in pH apparently did not have a detectable impact on the fall in cardiac output.

Glucocorticosteroids have been shown to stabilize lysosomal membranes and to preserve microcirculation. These effects could secondarily preserve myocardial function through decreased production of cardioinhibitory factors from ischemic organs such as the pancreas and small intestine. In the definitive swine study, no detectable improvement in hemodynamic parameters attributed to DEX were evident. Since glucocorticoids have a rather nonspecific overall effect on hemodynamics, cell metabolism, and organ function, it may be difficult to attribute an improvement in any one parameter to their action. Glucocorticoids ameliorate the decline in glucose concentrations in endotoxic shock (Yelich et al., 1987). In the swine studies, DEX was not able to prevent substantial declines in plasma glucose concentrations. The measurement of serum concentrations of lysosomal hydrolases might be a more appropriate method of assessing the effect of glucocorticoids.

The assessment of swine survival in these studies gave an early indication of the efficacy of SAC (in this case combined with magnesium sulfate) for lessening mortality due to parenterally administered T-2 toxin. The first dose of SAC was given immediately prior to T-2 toxin. Later rat studies confirmed the efficacy of SAC in animals parenterally exposed to T-2 toxin, although pretreatment 12 hr before toxin administration was necessary. The use of cathartics was not evaluated in the presently reported rat studies.

Despite the relatively small number of swine included in the definitive study, some degree of therapeutic efficacy was demonstrated for all drug combinations. It is necessary to reiterate that the dose of T-2 toxin given to these swine was quite high (3X LD_{50}). The shortcomings of the overall therapeutic approach taken might be overcome by optimizing the doses administered, as may be the case with metoclopramide, or selecting a more appropriate agent within a category of therapeutic action, such as the use of plasma expanders for maintenance of blood pressure instead of the crystalloid solution, normal saline.

In summary, a large number of drugs have been assessed for efficacy for the treatment of acute T-2 toxicosis. Many agents have not proven to be efficacious when used alone. It appears that the pathophysiology of acute T-2 toxicosis is too complex for a single agent to serve as the optimal therapy. Unfortunately, few studies have been conducted to assess therapeutic combinations. Such studies require large numbers of experimental animals and considerable resources. Obviously, the number of potential combinations of drug agents and administration protocols makes their assessment more difficult.

The ability to prevent early death following exposure to high doses T-2 toxin does not address the problems likely to be encountered several days later. Does the severe lymphoid necrosis result in seriously impaired

immune function requiring antibiotic therapy and isolation procedures? Are there delayed effects on organ function which have not been recognized? It is likely that a relativeiy long convalescent period would be required?

The present studies have also hopefully shed some light on ways in which T-2 toxin exerts its effects on experimental animals. The importance of enterohepatic recirculation of toxic metabolites on mortality has been better defined. In addition, toxic effects are probably due to both direct and indirect actions as was suggested by the effects of therapy on histologic lesion severity. Indirect effects are most likely a result of blood flow alterations and organ ischemia. The occurrence of a severe hypoglycemia in swine given T-2 toxin was also noted for the first time suggesting that reduced cell energy availability may contribute to lethality.

While the present work examined the efficacy of different therapeutic approaches for the treatment of toxicosis resulting from acute exposure to high amounts of the toxin, natural exposure to T-2 toxin would likely result in longer term exposure to much lower amounts. Circulatory shock would be unlikely to occur, thus there may be no need for fluid administration and base replacement. Glucocorticoids would be of little benefit under natural exposure conditions, and given the immunosuppressive effect of long-term administration of glucocorticoids, they might exacerbate ar already impaired immune system due to toxin exposure. The ability of SAC to improve survival in the present studies may have practical benefit following natural exposure to T-2 toxin and other trichothecenes. Oral administration of SAC would hasten elimination of the toxin and prevent enterohepatic recirculation, thereby lessening systemic exposure.

Given our present state of knowledge, current treatment recommendations for acute toxicosis include the early and repeated use of intestinal adsorbents such as SAC and the institution of a general supportive circulatory shock therapy including the use of high doses of glucocorticosteroids. Novel approaches to therapy such as the use of monoclonal antibodies should be investigated further. In addition, the role that cell receptor-toxin interactions play in the manifestation of toxic cellular effects of T-2 toxin should be examined with the hope that, if receptors are involved, an effective blocking agent could be found.

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APPENDIX I.A

Preliminary Swine Therapeutic Studies

A. Summary

Swine were treated iv with several different therapeutic agents either alone or in combinations following administration of 2.4 to 3.6 mg/kg T-2 toxin IV. Phenoxybenzamine, a nonspecific α-blocker, administered (1 mg/kg) by IV drip, appeared to improve peripheral perfusion and delay the onset of diarrhea in 2 swine compared to a positive T-2 control animal. Propranolol, a ß-blocker, hastened the decline in cardiac output and decreased the time to death in 2 swine given the drug at 0.15 mg/kg, as compared to a control animal. Survival time was enhanced in 2 pigs receiving either dexamethasone (8, 4, and 2 mg/kg) or methylprednisolone sodium succinate (15.2 mg/kg twice) along with bicarbonate and fluid therapy.

B. Materials and Methods

These preliminary studies were designed to allow for familiarization with surgical and monitoring techniques and to allow for formulation of an appropriate sampling protocol.

A summary of the preliminary swine treatment protocols is included in Table I.46. A more definitive materials and method discussion is included in Section M.2.

C. Results

Tables I.47 through I.54 have been included in this section providing data on several key parameters monitored during the conduct of these preliminary studies. All cardiac catheters implanted in Pig 5 (dopamine) were inoperative; therefore, it was decided to forgo in-depth physiological monitoring although the treatment protocol was continued. Animal survival times are noted in Table I.46.

Table I.46 Summary of preliminary swine therapeutic study.

Drug	Number of Animals and Survival Time	T-2 Dose	Treatment Protocol
Phenoxybenzamine, raw material courtesy of Smith, Kline, and French	n = 2 control = 1 All survived a 24-hour observation period prior to killing	2.4 mg/kg body weight (100 percent ETOH vehicle)*	1 mg/kg body weight administered by slow IV drip over 2-hour period. This was combined with a gravity drip of Ringer's solution.
Propranolol HCl injectable, Inderal® (1 mg propranolol/ml), Ayerst	n = 2** Mean survival time 4 hours	2.6 mg/kg body weight (100 percent ETOH vehicle)*	1 mg IV drip over first 5 minutes post-T-2, then 1 mg/min to reach a total dose of .15 mg/kg body weight, then a maintenance IV infusion of 0.5 mg/minute (1 animal received metoclopramide).
Dopamine HCl, Intropin® (40 mg dopamine/ml), American Critical Care	n = 2** Mean survival time 6 hours and 30 minutes	3.6 mg/kg body weight (100 percent ETOH vehicle)*	5 mcg/kg body weight/min via IV drip. If MAP*** declined, this rate was adjusted in an attempt to maintain adequate perfusion pressure (1 animal received Ringer's).
Dexamethasone Azium® (2 mg/ml) Schering	n = 1** Survival time 24 hours	3.6 mg/kg body weight (100 percent ETOH vehicle)*	8 mg/kg body weight IV immediately following T-2, then 4 mg/kg body weight IV at 4 hours and 2 mg/kg body weight at 8 hours. This was combined with sodium bicarbonate therapy and a gravity drip of Ringer's solution.
Methylprednisolone sodium succinate Solu-Medrol® (125 mg vial) Upjohn	n = 1** Survival time 23 hours	3.6 mg/kg body weight (100 percent ETOH vehicle)*	15.2 mg/kg body weight IV immediately following T-2 toxin and again at 4 hours post-T-2. This was combined with sodium bicarbonate therapy and a gravity drip of Ringer's solution.

*Total of .1 ml vehicle per kg body weight.
**One control animal was treated with 3.6 mg/kg body weight T-2 IV. The animal survived 6 hours and 5 minutes.
***Mean arterial blood pressure.

Table I.47 Preliminary swine therapeutic study: blood glucose concentrations.

Pig Number	Time 1 hour Pre	Glucose (mg/dl) 0	1	2	3	4	5	6	7	8	12	24	48
Preliminary T-2 Control	109	109	144	151	203	170	145						
P2ß-Blocker	108	120	122	101	85								
P3 MP*	108	100	88	106	85	64	46	33	38	37	52	43	
P4ß-Blocker	106	100	N/A	130	N/A	N/A							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	80	89	67	74	63	56	37	34	27	29	55	41	
P7 Dopamine	105	113	153	114	95	40							

N/A = not available
*MP = methylprednisolone
ß-Blocker = propranolol

Table I.48 Preliminary swine therapeutic study: total serum calcium/ionized serum calcium concentrations.

Pig Number	Time 1 hour Pre	Total Ca^{++}/Ionized Ca^{++} (mg/dl) 0	1	2	3	4	5	6	7	8	12	24	48
Preliminary T-2 Control	9.8/ 5.0	9.5/ 3.7	9.3/ 4.9	8.5/ 5.1	8.1/ 4.6	7.9/ 6.0	7.9/ 6.3						
P2ß-Blocker	9.1/ 5.3	8.1/ 5.3	9.0/ 5.5	9.0/ 5.3	8.8/ 5.4	8.2/ 4.9							
P3 MP*	9.4/ 5.4	9.1/ 3.9	8.6/ 2.2	8.1/ 5.0	7.5/ 5.1	7.5/ 5.3	7.4/ 6.1	7.3/ 5.5	7.2/ 5.8	7.1/ N/A	6.7/ 5.5	6.2/ 4.6	
P4ß-Blocker	9.6/ 6.1	9.4/ 6.1	10.0/ 6.3	8.5/ 5.1	8.4/ 5.2	8.3/ 4.8							
P5 Dopamine	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A					
P6 Azium	9.4/ 4.0	9.1/ 3.5	8.5/ 3.1	8.1/ 4.7	7.8/ 3.0	6.5/ 4.1	6.0/ 3.1	5.8/ 4.1	6.2/ 4.0	5.9/ 3.0	4.6/ 3.4	5.1/ 3.5	
P7 Dopamine	9.6/ 5.1	9.2/ 4.9	9.0/ 4.9	8.3/ 4.1	8.3/ 4.3	7.2/ 4.2							

N/A = not available.
*MP = Methylprednisolone.
ß-Blocker = propranolol.

Table I.49 Preliminary swine therapeutic study: heart rate.

Pig Number	Time 1 hour Pre	Heart Rate (Beats x Min^{-1}) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine	N/A	111	141	168	189	180	177	178	201	204	N/A	150	
Phenoxy-benzamine 2	N/A	66	135	147	168	168	147	162	171	174	N/A	108	
Phenoxy-benzamine	N/A 99	147	168	N/A	216	222	210	234	N/A	N/A	189		
Prelim-inary T-2 Control	90	138	84	120	144	218	214						
P2ß-Blocker	126	132	84	114	168								
P3 MP	120	114	N/A225		75	168	148	150	168	144	120	N/A	
P4ß-Blocker	112	108	111	84	180	198							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	96	90	114	114	114	108	120	138	141	132	147	231	
P7 Dopamine	90	118	99	96	174	234							

N/A = not available.
*MP = methylprednisolone.
ß-Blocker = propranolol.

Table I.50 Preliminary swine therapeutic study: cardiac index.

Pig Number	Time 1 hour Pre	Cardiac Index* (CO in ml x min^{-1} x kg^{-1})											
		0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine	N/A	168	163	160	153	199	150	147	121	120	N/A	131	
Phenoxy-benzamine	N/A	156	140	156	118	115	126	144	111	110	N/A	72	
Phenoxy-benzamine	N/A	125	131	153	N/A	79	72	83	84	93	N/A	103	
Prelim-inary T-2 Control	141	139	97	67	52	38	37	N/A					
P2ß-Blocker	183	156	99	59	31								
P3 MP**	156	144	N/A	80	80	115	115	146	95	163	97		
P4ß-Blocker	180	163	162	80	88	71							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	143	130	133	136	136	153	192	249	150	171	49	71	
P7 Dopamine	153	131	93	64	51	34							

N/A = not available.
*Cardiac index = cardiac output in ml x $minutes^{-1}$ x kg^{-1}.
**MP = methylprednisolone.
ß-Blocker = propranolol.

Table I.51 Preliminary swine therapeutic study: stroke volume.*

Pig Number	Time 1 hour Pre	Stroke Volume (ml · beat^{-1} x kg^{-1}) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A	1.51	1.16	.95	.81	1.11	.85	.83	.60	.59	N/A	.87	
Phenoxy-benzamine 2	N/A	2.36	1.04	1.06	.70	.68	.86	.89	.65	.63	N/A	.54	
Phenoxy-benzamine	N/A	1.26	.89	.91	N/A	.37	.32	.40	.36	N/A	N/A	.54	
Prelim-inary T-2 Control	1.57	1.01	1.15	.56	.36	.17	.17						
P2ß-Blocker	1.45	1.13	1.18	.52	.18								
P3 MP**	1.30	1.26	N/A	.70	1.07	.68	.78	.97	.57	1.13	.81	N/A	
P4ß-Blocker	1.61	1.51	1.46	.95	.49	.44							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	1.49	1.44	1.17	1.19	1.19	1.42	1.60	1.80	1.06	1.30	.33	.31	
P7 Dopamine	1.70	1.11	.94	.67	.29	.15							

N/A = not available.
*Stroke volume = heart rate (beats · min^{-1}) divided by cardiac index (ml · min^{-1} · kg^{-1}).
**MP = methylprednisolone.
ß-Blocker = propranolol.

Table I.52 Preliminary swine therapeutic study: mean arterial blood pressure.

Pig Number	Time 1 hour Pre	Mean Arterial Pressure (mmHg) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A	120	104	76	62	52	51	45	48	46	N/A	80	
Phenoxy-benzamine 2	N/A	109	100	76	64	56	54	60	48	52	N/A	60	
Phenoxy-benzamine	N/A	120	112	96	N/A	69	58	56	54	N/A	N/A	68	
Prelim-inary T-2 Control	130	115	97	67	52	38	37						
P2ß-Blocker	124	100	124	116	73								
P3 MP*	N/A due to inoperative catheter												
P4ß-Blocker	102	109	94	62	52	58							
P5 Dopamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A					
P6 Azium	124	122	112	110	58	60	64	67	72	78	114	112	
P7 Dopamine	116	116	100	92	84	72							

N/A = not available.
*MP = methylprednisolone.
ß-Blocker = propranolol.

Table I.53 Preliminary swine therapeutic study: arterial blood pH/lactic acid determinations.

Pig Number	Time 1 hour Pre	Arterial Blood pH/Lactic Acid (mmoles/L) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A/ N/A	7.435/ 1.6	7.407/ 2.9	7.372/ 3.8	7.305/ 2.5	7.255/ 4.2	7.244/ 6.3	7.226/ 3.6	7.250/ 0.9	7.336/ 2.3	N/A/ N/A	7.391/ 1.5	
Phenoxy-benzamine 2	N/A/ N/A	7.428/ 2.6	7.316/ 4.7	7.269/ 2.4	7.279/ 2.1	7.252/ 4.6	7.292/ 4.3	7.301/ 5.5	7.283/ 5.2	7.261/ 5.8	N/A/ N/A	7.324/ 3.1	
Phenoxy-benzamine	N/A/ N/A	7.428/ N/A	7.307/ N/A	7.336/ N/A	N/A/ N/A	7.285/ N/A	7.289/ N/A	7.312/ N/A	7.328/ N/A	7.320/ N/A	N/A/ N/A	7.437/ N/A	
Preliminary T-2 Control**	/.85	/1.10	/3.14	/3.02	/5.58	/7.87	/9.08	/16.51					
P2ß-Blocker**	/1.71	/1.45	/2.83	/3.72	/6.56								
P3 MP*	/1.53	/1.56	N/A	/3.48	/2.91	/3.14	/3.98	/4.05	/3.86	/3.38	/2.35	N/A	
P4ß-Blocker**	/.60	/.57	/2.10	/2.91	/3.05	/6.41							
P5 Dopamine**	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A					
P6 Azium**	/.86	/1.05	/1.55	/1.75	/1.65	/1.87	/1.40	/2.10	/2.51	/2.25	/1.89	/4.88	
P7 Dopamine**	N/A/ N/A	/.84	/7.61	/3.30	/4.12	/6.69							

N/A = not available.
*MP = methylprednisolone.
** = blood pH data not available due to malfunctioning blood-gas machine.
ß-Blocker = propranolol.

Table I.54 Preliminary swine therapeutic study: arterial blood gas determinations.

Pig Number	Time 1 hour Pre	Arterial Blood Gases $PaO_2/PaCO_2$ (mmHg) 0	1	2	3	4	5	6	7	8	12	24	48
Phenoxy-benzamine 1	N/A/ N/A	77.4 45.1	N/A N/A	86.3/ 19.0	96.8/ 37.7	94.2/ 40.7	101.4/ 35.4	92.3/ 37.7	84.6/ 34.0	97.9/ 26.5	N/A/ N/A	99.7/ 26.6	
Phenoxy-benzamine 2	N/A/ N/A	96.5/ 36.1	99.7/ 41.5	91.6/ 34.8	87.2/ 46.6	94.0/ 45.3	101.0/ 39.1	97.8/ 37.5	98.7/ 37.6	99.1/ 37.1	N/A/ N/A	94.2/ 34.1	
Phenoxy-benzamine	N/A/ N/A	99.2/ 39.2	105.9/ 35.3	99.8/ 34.4	N/A/ N/A	101.7/ 31.0	106.1/ 29.7	111.7/ 25.2	104.7/ 21.6	106.5/ 27.2	N/A/ N/A	N/A/ 25.1	
Prelim-inary T-2 Control	98.7/ 35.0	98.7/ 36.9	100.1/ 34.6	101.5/ 31.8	106.6/ 29.9	106.0/ 28.7	119.7/ 23.0	104.6/ 33.6					
P2ß-Blocker	91.2/ 36.0	92.8/ 35.4	97.4/ 37.3	72.5/ 41.8	60.3/ 39.7								
P3 MP*	94.8/ 38.5	75.7/ 33.4	N/A/ N/A	81.9/ 32.0	86.8/ N/A	82.0/ N/A	95.6/ N/A	90.0/ N/A	98.8/ 29.5	95.0/ 38.0	91.8/ 28.2		
P4ß-Blocker	94.3/ 35.7	82.7/ 40.7	95.5/ 34.7	72.5/ 36.7	75.6/ 36.2	67.0/ 30.5							
P5 Dopamine	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A	N/A/ N/A					
P6 Azium	109.4/ 37.4	106.1/ 33.9	99.6/ 32.4	91.0/ 36.0	99.4/ 32.2	97.1/ 36.8	103.0/ 38.6	107.8/ 36.9	95.9/ 37.0				
P7 Dopamine	N/A/ N/A	90.6/ 41.2	89.9/ 43.3	88.5/ 41.8	82.6/ 39.5	104.9/ 30.9							

N/A = not available.
*MP = methylprednisolone.
ß-Blocker = propranolol.

APPENDIX I.B

Table I.55 Hemodynamic values ± SEM.

	Hours Postdosing									
	-1	0	1	2	3	4	5	6	7	8
Cardiac Index (ml x min^{-1} x kg^{-1})										
No therapy	176.23 ± 7.88	152.64 ± 11.74	190.14 ± 36.73	189.43 ± 35.90	159.09 ± 22.86	132.63 ± 14.08	103.21 ± 12.83	96.92 ± 7.45	101.02 ± 12.07	82.15 ± 14.65
No A.C.	183.66 ± 16.85	152.79 ± 12.46	187.47 ± 14.19	199.12 ± 6.10	213.61 ± 23.07	193.54 ± 21.79	181.50 ± 17.95	180.52 ± 8.33	184.51 ± 8.67	190.40 ± 30.87
No HCO_3	170.52 ± 15.18	133.30 ± 6.21	172.34 ± 26.70	179.77 ± 27.80	205.78 ± 32.58	205.60 ± 27.71	226.28 ± 50.85	178.33 ± 29.53	173.73 ± 30.09	134.38 ± 29.76
No saline	159.48 ± 15.56	160.77 ± 13.93	156.52 ± 8.77	159.83 ± 7.42	134.94 ± 7.15	138.44 ± 8.13	135.36 ± 11.07	132.95 ± 10.74	118.80 ± 8.29	133.51 ± 12.51
All therapy	197.29 ± 53.66	213.42 ± 50.24	233.20 ± 51.04	217.20 ± 42.10	202.86 ± 39.57	185.44 ± 37.47	203.83 ± 40.50	192.59 ± 34.39	178.48 ± 46.58	178.81 ± 61.19
Heart Rate (beats x min^{-1})										
No therapy	101.00 ± 7.81	97.00 ± 8.72	100.00 ± 1.27	133.00 ± 18.03	186.00 ± 6.00	193.00 ± 13.89	216.00 ± 10.54	198.00 ± 20.42	213.00 ± 19.97	192.00 ± N/A
No A.C.	126.00 ± 12.49	111.00 ± 9.17	122.00 ± 13.45	152.00 ± 28.58	159.00 ± 23.30	215.00 ± 4.36	205.00 ± 12.53	192.00 ± 8.66	192.00 ± 5.20	183.00 ± 11.36
No HCO_3	101.25 ± 4.31	97.50 ± 1.94	108.75 ± 3.75	107.25 ± 4.80	133.50 ± 1.94	146.25 ± 4.48	152.25 ± 12.69	150.00 ± 11.22	143.25 ± 9.03	147.75 ± 12.93
No saline	104.00 ± 8.72	104.00 ± 8.89	99.00 ± 9.17	114.00 ± 1.73	130.00 ± 7.81	170.00 ± 5.67	161.00 ± 9.85	158.00 ± 8.72	174.00 ± 3.00	169.00 ± 2.65
All therapy	97.80 ± 10.46	95.40 ± 8.24	106.20 ± 11.83	123.60 ± 8.24	131.40 ± 9.22	163.20 ± 8.19	158.20 ± 10.96	159.00 ± 5.02	157.60 ± 3.78	166.80 ± 6.68
Left Atrial Pressure (mmHg)										
No therapy	3.67 ± 1.33	3.33 ± 0.33	1.33 ± 1.20	-2.33 ± 1.76	-4.67 ± 1.20	-0.67 ± 2.96	-3.67 ± 2.03	-1.67 ± 1.45	-3.33 ± 0.67	-4.00 ± 0.00
No A.C.	7.00 ± 3.61	7.33 ± 2.19	4.33 ± 2.33	0.33 ± 0.33	1.67 ± 1.20	4.00 ± 2.08	5.00 ± 1.53	2.67 ± 2.19	9.66 ± N/A	4.00 ± 1.00
No HCO_3	6.00 ± 3.76	9.33 ± 2.85	10.67 ± 2.73	1.33 ± 1.86	4.75 ± 1.65	6.00 ± 3.00	5.25 ± 0.85	6.00 ± 1.15	6.25 ± 0.48	3.50 ± 1.89
No saline	5.00 ± 1.00	3.33 ± 1.20	2.33 ± 0.33	-0.67 ± 0.88	0.33 ± 0.33	0.33 ± 0.67	0.67 ± 0.33	0.67 ± 0.33	1.33 ± 0.88	2.33 ± 0.88
All therapy	4.60 ± 1.25	4.40 ± 0.93	3.80 ± 1.59	2.00 ± 1.22	1.60 ± 1.03	2.60 ± 1.69	2.00 ± 1.52	2.40 ± 1.81	2.80 ± 1.88	4.00 ± 1.96
Left Ventricular Work (kg x m x min^{-1} x kg^{-1})										
No therapy	295.48 ± 33.37	226.74 ± 20.19	268.27 ± 45.41	237.04 ± 32.36	154.20 ± 18.27	108.41 ± 13.12	70.15 ± 6.77	71.74 ± 8.97	7[illegible].07 ± 10.49	59.81 ± 13.91
No A.C.	287.30 ± 42.58	230.01 ± 25.97	265.75 ± 18.41	239.05 ± 22.49	190.13 ± 33.70	148.36 ± 15.03	152.55 ± 16.57	152.86 ± 8.40	165.89 ± 18.42	178.45 ± 36.20
No HCO_3	291.39 ± 14.62	218.04 ± 3.75	300.05 ± 56.17	255.21 ± 51.66	252.09 ± 55.84	192.27 ± 41.30	219.19 ± 68.72	155.18 ± 45.00	156.23 ± 47.03	115.99 ± 20.18
No saline	N/A	242.08 ± 13.77	234.62 ± 6.08	207.32 ± 12.69	129.11 ± 10.78	110.06 ± 6.71	103.52 ± 12.34	95.28 ± 9.94	82.18 ± 12.05	97.76 ± 8.87
All therapy	280.06 ± 73.17	306.84 ± 55.51	324.72 ± 66.04	299.70 ± 47.21	216.69 ± 37.95	162.54 ± 28.84	164.71 ± 29.30	129.83 ± 25.01	117.22 ± 27.86	119.82 ± 35.02
Pulmonary Arterial Pressure (mmHg)										
No therapy	16.67 ± 2.19	14.33 ± 0.88	12.00 ± 0.58	17.33 ± 4.37	17.00 ± 5.13	18.00 ± 3.00	13.33 ± 1.45	13.00 ± 1.53	11.33 ± 0.33	11.00 ± 1.00
No A.C.	20.67 ± 3.28	18.00 ± 2.52	23.33 ± 6.17	22.67 ± 4.06	20.00 ± 0.58	19.33 ± 0.67	19.00 ± 0.58	21.00 ± 2.31	21.33 ± 1.86	22.00 ± 3.06
No HCO_3	21.00 ± 1.08	18.33 ± 1.67	24.33 ± 6.36	25.50 ± 4.99	23.50 ± 4.63	24.67 ± 3.28	21.00 ± 3.06	21.33 ± 2.85	21.00 ± 2.65	21.00 ± 3.21
No saline	17.33 ± 1.45	16.33 ± 1.45	14.33 ± 0.67	19.67 ± 1.20	21.00 ± 2.52	20.00 ± 3.61	17.00 ± 1.15	15.67 ± 1.86	15.67 ± 0.88	16.67 ± 0.67
All therapy	15.60 ± 1.21	16.80 ± 1.02	17.60 ± 2.91	22.40 ± 3.71	23.40 ± 3.91	26.20 ± 3.48	19.20 ± 1.50	17.60 ± 1.75	15.20 ± 1.56	16.25 ± 1.65

Table I.55 continued

	Hours Postdosing																	
	-1		0		1		2		3		4		5		6		7		8	
Pulmonary Vascular Resistance (mmHg/ml x min^{-1} x kg^{-1})																				
No therapy	0.07	± 0.01	0.07	± 0.01	0.06	± 0.01	0.12	± 0.05	0.15	± 0.05	0.15	± 0.03	0.17	± 0.02	0.15	± 0.01	0.15	± 0.01	0.19	± 0.05
No A.C.	0.08	± 0.01	0.07	± 0.01	0.10	± 0.02	0.11	± 0.02	0.09	± 0.01	0.08	± 0.01	0.08	± 0.01	0.10	± 0.01	N/A		0.09	± 0.03
No HCO_3	0.11	± 0.02	0.10	± 0.00	0.21	± 0.00	0.14	± 0.11	0.11	± 0.05	0.14	± 0.05	0.11	± 0.04	0.18	± 0.00	0.12	± 0.06	0.16	± 0.06
No saline	0.08	± 0.01	0.08	± 0.01	0.08	± 0.00	0.13	± 0.01	0.15	± 0.02	0.14	± 0.02	0.12	± 0.01	0.11	± 0.01	0.12	± 0.01	0.11	± 0.01
All therapy	0.07	± 0.01	0.07	± 0.01	0.07	± 0.02	0.11	± 0.03	0.13	± 0.04	0.15	± 0.04	0.10	± 0.03	0.09	± 0.30	0.10	± 0.02	0.08	± 0.02
Rate Pressure Product																				
No therapy	148.49	± 3.41	127.08	± 13.13	132.54	± 14.42	166.62	± 29.39	212.96	± 4.90	203.04	± 14.18	185.68	± 23.07	185 64	± 23.74	210.38	± 14.09	184.32	± N/A
No A.C.	170.76	± 9.00	144.89	± 10.09	158.80	± 14.67	185.84	± 37.59	174.64	± 31.03	209.46	± 16.47	207.06	± 13.24	184.10	± 9.28	176.32	± 13.00	185.80	± 29.92
No HCO_3	154.71	± 7.11	145.14	± 1.94	164.80	± 6.63	146.05	± 8.18	163.36	± 15.90	164.49	± 12.03	173.97	± 26.05	152.28	± 18.53	159.57	± 16.49	157.78	± 24.49
No saline	146.58	± 11.90	140.10	± 12.64	138.58	± 17.00	138.32	± 10.82	144.78	± 10.34	149.00	± 8.43	136.96	± 10.33	120.80	± 10.67	130.24	± 12.91	151.28	± 16.24
All therapy	126.84	± 18.46	127.61	± 8.27	143.14	± 15.73	159.34	± 12.08	144.41	± 12.30	173.08	± 17.87	159.73	± 16.34	142.99	± 11.36	147.50	± 16.84	164.99	± 22.36
Right Ventricular Work (kg x mm x min^{-1} x kg^{-1})																				
No therapy	39.48	± 3.24	29.48	± 0.75	31.40	± 7.34	41.93	± 6.80	35.12	± 7.91	32.74	± 7.24	19.04	± 4.22	17.31	± 3.07	15.62	± 2.10	12.09	± 1.07
No A.C.	52.63	± 12.61	37.64	± 6.68	59.00	± 16.06	60.89	± 9.44	57.79	± 4.74	50.95	± 6.41	47.00	± 5.44	51.03	± 3.31	53.09	± 1.95	57.67	± 12.64
No HCO_3	49.96	± 7.25	37.95	± 0.13	52.35	± 16.21	56.87	± 10.31	57.86	± 10.98	61.09	± 6.19	54.39	± 6.03	45.12	± 2.00	41.63	± 1.08	34.57	± 0.44
No saline	37.71	± 4.97	36.24	± 6.17	30.65	± 2.97	42.96	± 4.47	38.74	± 5.82	38.37	± 8.94	31.56	± 4.26	28.81	± 5.38	25.49	± 3.00	30.24	± 2.86
All therapy	38.97	± 7.14	46.14	± 7.11	52.00	± 8.68	64.42	± 12.39	58.48	± 6.50	62.25	± 9.55	53.36	± 11.18	45.08	± 7.94	38.31	± 9.04	39.03	± 11.80
Stroke Volume (ml x $beat^{-1}$ x min^{-1})																				
No therapy	1.77	± 0.19	1.58	± 0.04	1.92	± 0.36	1.51	± 0.44	0.87	± 0.15	0.70	± 0.12	0.49	± 0.08	0.50	± 0.07	0.48	± 0.08	0.50	± 0.00
No A.C.	1.51	± 0.29	1.38	± 0.08	1.55	± 0.07	1.41	± 0.28	1.43	± 0.33	0.90	± 0.09	0.89	± 0.11	0.95	± 0.08	0.96	± 0.00	1.06	± 0.21
No HCO_3	1.62	± 0.13	1.36	± 0.03	1.63	± 0.25	1.69	± 0.20	1.53	± 0.24	1.41	± 0.20	1.40	± 0.27	1.13	± 0.21	1.19	± 0.25	0.88	± 0.20
No saline	1.54	± 0.11	1.57	± 0.19	1.61	± 0.19	1.40	± 0.08	1.04	± 0.02	0.82	± 0.06	0.85	± 0.11	0.85	± 0.11	0.68	± 0.06	0.79	± 0 08
All therapy	1.99	± 0.45	2.21	± 0.44	2.26	± 0.51	1.78	± 0.34	1.58	± 0.36	1.15	± 0.25	1.30	± 0.24	1.21	± 0.20	1.10	± 0.27	1.08	± 0.33
Total Peripheral Resistance (mmHg/ml x min^{-1} x kg^{-1})																				
No therapy	0.70	± 0.03	0 72	± 0.05	0.60	± 0.12	0.55	± 0.14	0.48	± 0.09	0.46	± 0.05	0.51	± 0.08	0.56	± 0.02	0.52	± 0.05	0.66	± C.08
No A.C.	0.63	± 0.04	0.73	± 0.06	0.57	± 0.06	0.44	± 0.02	0.31	± 0.03	0.30	± 0.04	0.35	± 0.03	0.35	± 0.03	0.36	± 0.03	0.37	± 0.05
No HCO_3	0.76	± 0.11	0.91	± 0.08	0.77	± 0.10	0.59	± 0.06	0.44	± 0.06	0.34	± 0.04	0.32	± 0.04	0.36	± 0.05	0.38	± 0.05	0.50	± 0.09
No saline	N/A		0.71	± 0.09	0.71	± 0.07	0.60	± 0.03	0.54	± 0.09	0.43	± 0.04	0.42	± 0.03	0.40	± 0.04	0.43	± 0.05	0.41	± 0.05
All therapy	0.64	± 0.11	0.62	± 0.12	0.52	± 0.09	0.54	± 0.08	0.44	± 0.07	0.41	± 0.09	0.35	± 0.07	0.33	± 0.07	0.32	± 0.07	0.47	± 0.21

N/A = not available.

Table I.56 Blood-gas values ± SEM.

	Hours Postdosing																					
	-1		0		1		2		3		4		5		6		7		8			
Anion Gap (mEq/L)																						
No therapy	63.89	1.94	65.16	3.20	65.21	9.50	52.24	1.15	49.21	3.06	47.23	1.04	42.60	1.67	42.45	2.22	43.30	1.92	41.53	2.32		
No A.C.	63.84	1.08	63.51	1.40	53.82	1.73	50.21	1.58	50.50	2.25	48.22	1.47	51.27	0.77	53.64	3.45	52.33	2.15	56.13	4.58		
No HCO_3	67.42	1.01	66.59	0.82	56.71	2.31	51.85	4.04	42.77	4.65	42.99	5.21	40.83	4.29	55.55	5.77	38.79	4.50	46.90	9.31		
No saline	66.52	5.72	65.06	4.36	57.29	2.72	55.14	1.54	53.74	1.40	53.21	0.72	56.04	4.89	46.99	8.53	57.59	1.90	60.90	3.00		
All therapy	67.34	1.92	66.61	1.40	59.45	0.97	54.82	2.35	49.78	2.22	47.14	1.63	46.95	2.10	45.58	2.41	52.15	2.80	47.39	2.30		
Arterial Bicarbonate (mEq/L)																						
No therapy	24.23	2.31	23.16	1.87	21.21	1.93	17.90	0.61	16.21	0.88	14.56	0.61	14.26	0.85	13.45	0.80	12.96	0.79	10.87	1.00		
No A.C.	23.17	1.67	23.51	1.75	18.82	1.21	17.88	0.98	18.38	0.72	17.22	0.19	19.27	0.83	20.31	0.41	19.33	0.22	19.47	1.41		
No HCO_3	26.92	0.50	26.09	0.46	19.96	1.09	18.35	0.43	16.02	0.34	15.49	0.32	14.83	0.49	15.30	0.37	14.83	0.49	15.15	0.47		
No saline	25.18	0.72	25.39	0.76	21.62	0.90	19.80	0.21	18.74	0.57	19.21	1.17	19.37	1.77	16.98	1.54	19.92	0.63	20.24	0.34		
All therapy	27.14	0.63	26.81	0.55	22.45	0.42	20.02	0.48	18.58	0.87	18.34	0.44	18.15	1.00	19.18	1.03	20.55	1.01	17.39	2.44		
Arterial Blood pH (pH units)																						
No therapy	7.42	0.04	7.40	0.04	7.39	0.04	7.30	0.02	7.24	0.02	7.22	0.01	7.22	0.03	7.21	0.03	7.21	0.03	7.10	0.06		
No A.C.	7.39	0.03	7.40	0.03	7.31	0.04	7.30	0.03	7.28	0.02	7.27	0.01	7.31	0.02	7.35	0.03	7.34	0.02	7.35	0.03		
No HCO_3	7.47	0.01	7.45	0.01	7.37	0.01	7.32	0.01	7.26	0.01	7.25	0.01	7.22	0.02	7.26	0.02	7.28	0.03	7.31	0.02		
No saline	7.45	0.01	7.45	0.00	7.38	0.01	7.35	0.01	7.32	0.02	7.32	0.03	7.33	0.03	7.35	0.03	7.38	0.02	7.40	0.01		
All therapy	7.47	0.01	7.46	0.01	7.40	0.01	7.35	0.01	7.31	0.01	7.30	0.01	7.31	0.02	7.33	0.02	7.38	0.02	7.29	0.09		
Arterial Carbon Dioxide Tension (mmHg)																						
No therapy	37.73	1.29	37.80	0.10	34.13	1.48	35.80	0.90	37.33	0.88	32.73	1.68	30.83	1.13	29.00	2.10	26.37	3.21	25.93	4.16		
No A.C.	38.73	1.56	37.97	0.35	37.87	1.21	35.77	0.75	40.57	1.96	37.83	1.11	38.40	1.46	37.20	3.55	35.17	2.30	34.37	1.99		
No HCO_3	37.32	0.87	38.45	1.45	33.40	2.75	34.42	1.93	34.90	0.45	33.37	0.37	34.45	1.47	31.55	1.79	27.30	1.92	25.15	0.89		
No saline	36.60	2.06	36.57	1.40	35.83	0.18	34.97	1.47	35.80	1.80	38.03	0.32	35.90	3.46	32.33	2.12	31.47	1.64	30.87	0.45		
All therapy	38.22	0.37	38.52	0.68	36.12	1.33	35.44	1.24	36.04	1.86	36.86	0.92	35.08	2.79	35.18	1.92	33.64	1.10	35.62	3.80		
Arterial Oxygen Tension (mmHg)																						
No therapy	99.70	2.56	97.03	1.03	104.10	0.31	100.57	0.19	98.53	1.32	108.40	2.01	170 10	1.01	109.20	2.31	113.67	0.75	109.73	9.38		
No A.C.	95.97	6.12	97.33	2.34	96.80	3.57	102.27	1.40	91.77	6.09	88.53	3.02	88.77	4.89	90.37	2.88	95.03	1.91	92.17	4.59		
No HCO_3	93.85	1.02	95.40	2.09	98.80	2.64	96.85	2.34	95.87	1.76	94.82	4.77	103.00	1.05	101.42	1.98	107.82	3.21	108.40	1.86		
No saline	98.47	3.16	97.63	3.88	98.67	0.94	98.23	2.69	91.70	3.61	89.23	1.40	94 80	2.71	92.07	2.00	94.57	0.52	97.90	3.35		
All therapy	97.70	1.12	96.10	2.51	102.10	2.24	97.64	1.01	92.14	2.51	94.48	1.87	98.14	2.98	98.48	1.65	96.06	4.09	82.57	12.07		

ble I.56 continued

	Hours Postdosing																	
	-1		0		1		2		3		4		5		6		7		8	
dy Temperature (°C)																				
o therapy	39.50	± 0.21	39.57	± 0.43	39.07	± 0.03	39.23	± 0.03	39.50	± 0.15	40.13	± 0.09	40.17	± 0.07	40.20	± 0.06	40.27	± 0.07	40.40	± 0.12
o A.C.	39.33	± 0.28	40.13	± 0.48	39.87	± 0.47	39.77	± 0.52	39.37	± 0.35	39.10	± 0.15	38.87	± 0.32	38.43	± 0.47	38.27	± 0.54	38.07	± 0.97
o HCO_3	39.30	± 0.18	39.92	± 0.37	39.32	± 0.25	39.20	± 0.19	39.07	± 0.11	38.97	± 0.14	38.90	± 0.14	38.65	± 0.06	38.52	± 0.19	38.47	± 0.15
o saline	39.13	± 0.20	39.47	± 0.32	39.17	± 0.20	39.33	± 0.27	39.43	± 0.38	39.70	± 0.26	39.43	± 0.42	39.17	± 0.34	38.77	± 0.20	38.73	± 0.15
ll therapy	39.18	± 0.07	39.48	± 0.19	39.14	± 0.16	39.00	± 0.11	38.94	± 0.17	39.04	± 0.15	38.66	± 0.27	38.46	± 0.24	38.04	± 0.39	38.02	± 0.42
ctic Acid (mmol/liter)																				
o therapy	0.78	± 0.04	0.77	± 0.06	1.50	± 0.27	2.00	± 0.34	2.11	± 0.37	3.26	± 0.53	3.75	± 0.99	4.16	± 0.80	5.18	± 0.91	8.74	± 1.25
o A.C.	0.59	± 0.33	0.94	± 0.68	2.27	± 0.59	1.59	± 0.51	1.50	± 0.31	1.52	± 0.37	1.86	± 0.32	1.51	± 0.03	1.35	± 0.13	3.74	± 2.63
o HCO_3	1.17	± 0.32	0.93	± 0.15	2.04	± 0.23	1.89	± 0.24	1.81	± 0.11	1.89	± 0.17	2.39	± 0.45	1.80	± 0.37	1.94	± 0.42	1.51	± 0.45
o saline	0.90	± 0.11	0.93	± 0.17	2.19	± 0.20	2.32	± 0.51	1.82	± 0.18	1.81	± 0.12	2.06	± 0.12	2.07	± 0.56	1.96	± 0.31	2.73	± 0.24
ll therapy	1.03	± 0.20	1.17	± 0.29	1.76	± 0.25	1.83	± 0.32	1.78	± 0.40	2.25	± 0.45	2.35	± 0.46	2.22	± 0.55	2.12	± 0.39	5.87	± 3.22

Table 1.57 Hematology values ± SEM

	Hours Postdosing																	
	-1		0		1		2		3		4		5		6		7		8	
Absolute Lymphocyte Count (x 10^3/μl)																				
No therapy	7.06	1.07	6.03	0.41	12.93	0.93	10.07	1.76	9.22	1.08	6.25	0.75	5.33	1.11	6.51	2.93	3.84	1.24	3.36	1.18
No A.C.	6.89	0.98	4.57	0.40	8.17	0.99	7.50	0.89	6.73	0.35	4.69	0.68	3.98	1.27	2.32	0.41	1.92	0.71	1.44	0.53
No HCO_3	6.07	0.53	4.49	0.22	9.52	0.63	7.97	0.25	7.25	0.74	5.78	0.41	3.29	0.36	2.44	0.17	2.34	0.22	1.86	0.34
No saline	6.30	1.11	4.06	0.47	11.54	0.86	10.87	1.36	8.74	0.28	5.85	0.28	3.85	0.45	2.82	0.37	1.87	0.34	1.58	0.40
All therapy	5.98	0.60	4.45	0.47	7.00	0.94	7.82	0.87	6.68	0.45	14.22	9.42	3.65	0.52	2.71	0.45	2.40	0.44	2.41	0.38
Absolute Neutrophil Count (x 10^3/μl)																				
No therapy	12.04	2.21	11.41	1.43	12.40	3.23	9.74	2.25	1.19	0.42	0.28	0.11	0.93	0.40	4.15	1.49	3.26	0.33	4.53	0.72
No A.C.	4.54	1.11	7.31	1.69	5.32	2.49	3.43	1.65	1.88	1.12	0.90	0.50	1.87	1.17	3.03	1.60	3.27	1.64	5.23	2.22
No HCO_3	9.49	1.63	8.97	2.57	13.86	3.73	4.09	1.58	4.56	2.19	1.24	0.19	2.53	1.18	3.60	1.26	5.10	1.97	4.85	1.50
No saline	6.21	1.37	8.27	2.29	13.36	3.70	10.50	1.25	6.65	5.25	1.01	0.66	1.62	0.90	1.86	0.19	3.72	0.83	4.68	1.00
All therapy	6.69	1.08	8.74	2.02	10.45	2.75	6.50	1.74	5.92	1.68	1.41	0.45	1.12	0.70	2.11	1.40	3.17	1.25	6.26	3.59
Corrected White Blood Cell Count (x 10^3/μl)																				
No therapy	20.63	3.03	19.26	3.23	28.00	4.71	21.50	3.24	11.63	0.29	6.84	0.75	6.52	1.57	11.39	4.84	7.93	1.52	9.39	2.20
No A.C.	11.93	2.14	12.77	2.28	13.94	2.76	11.08	2.35	9.80	2.16	5.79	0.77	6.07	1.57	5.58	1.42	5.54	1.68	6.94	2.31
No HCO_3	16.53	1.91	15.02	3.46	25.42	3.87	13.37	1.74	13.05	2.56	7.47	0.44	6.09	1.01	6.19	1.40	7.92	2.47	7.11	1.93
No saline	13.37	1.47	12.93	1.93	26.63	2.92	22.78	1.69	16.92	5.47	7.01	0.71	5.72	1.08	4.96	0.39	5.79	1.00	6.85	1.51
All therapy	13.32	0.87	14.06	2.15	18.20	2.82	14.70	2.52	13.92	2.49	16.17	9.92	4.97	1.26	5.13	1.57	5.95	1.75	9.55	4.12
Hematocrit (%)																				
No therapy	29.00	0.58	27.00	0.58	35.33	0.88	37.50	1.26	37.50	1.26	36.00	2.00	37.00	1.15	37.00	1.53	39.33	1.67	40.33	1.20
No A.C.	27.33	1.45	26.83	1.36	32.33	1.76	32.67	2.40	29.33	2.19	28.50	2.02	28.33	1.86	29.00	1.53	28.33	1.86	29.67	1.45
No HCO_3	30.75	1.31	29.50	0.96	36.00	1.47	37.00	1.08	35.25	0.25	33.75	1.25	32.38	1.60	32.25	1.60	32.75	2.39	34.00	2.55
No saline	29.33	1.20	27.33	1.20	35.67	1.45	37.50	0.87	35.83	1.17	32.67	0.88	33.00	0.00	32.67	0.33	31.83	1.09	32.67	1.45
All therapy	28.70	1.48	27.70	0.97	34.20	1.74	35.80	1.24	34.20	1.32	31.40	1.21	30.20	0.97	29.20	1.24	31.20	1.62	30.50	1.43
Hemoglobin (gm/dl)																				
No therapy	9.40	0.10	8.90	0.30	11.30	0.36	12.37	0.43	12.50	0.23	11.73	0.39	12.07	0.43	11.70	0.10	12.13	0.20	13.20	0.17
No A.C.	8.87	0.33	8.80	0.20	10.33	0.18	10.33	0.60	9.17	0.49	8.67	0.35	8.90	0.38	9.27	0.23	9.13	0.35	9.13	0.52
No HCO_3	9.97	0.56	9.60	0.32	11.55	0.60	12.07	0.59	11.20	0.21	10.65	0.34	10.27	0.54	10.30	0.44	10.60	0.59	10.95	0.65
No saline	9.60	0.31	9.07	0.43	11.97	0.62	12.47	0.37	12.07	0.57	10.77	0.38	10.80	0.20	10.67	0.27	10.60	0.35	10.90	0.46
All therapy	9.12	0.59	9.02	0.53	10.74	0.64	11.62	0.52	11.04	0.60	10.16	0.49	9.52	0.36	9.40	0.50	10.10	0.69	9.82	0.47

Table 1.57 continued

	Hours Postdosing																	
	-1		0		1		2		3		4		5		6		7		8	
Nucleated Red Blood Cells (per 100 wbc)																				
No therapy	0.33	± 0.33	0.33	± 0.33	0.00	± 0.00	0.67	± 0.33	0.00	± 0.00	1.33	± 0.88	8.33	± 4.26	10.33	± 5.04	13.67	± 9.21	11.67	± 5.49
No A.C.	0.00	± 0.00	0.00	± 0.00	0.00	± 0.00	1.67	± 0.33	4.00	± 3.06	9.33	± 4.70	10.67	± 2.67	14.67	± 3.18	23.00	± 11.68	25.33	± 15.51
No HCO_3	0.25	± 0.25	0.00	± 0.00	0.50	± 0.50	0.75	± 0.48	5.25	± 4.03	2.75	± 1.70	4.75	± 2.63	17.00	± 5.03	10.25	± 3.88	15.00	± 7.33
No saline	0.00	± 0.00	0.00	± 0.00	0.00	± 0.00	0.33	± 0.33	0.67	± 0.33	6.00	± 2.08	7.33	± 2.33	10.00	± 1.00	12.67	± 0.33	10.67	± 4.98
All therapy	0.20	± 0.20	0.00	± 0.00	1.20	± 0.80	1.00	± 0.77	7.00	± 2.49	7.80	± 3.29	15.00	± 5.57	8.80	± 1.66	11.60	± 2.79	8.40	± 1.89
Red Blood Cell Count (x 10^6 µl)																				
No therapy	6.01	± 0.43	5.69	± 0.60	7.63	± 0.69	8.14	± 0.60	8.58	± 0.81	7.18	± 0.79	7.24	± 0.18	7.34	± 0.51	7.36	± 0.27	8.07	± 0.29
No A.C.	5.76	± 0.49	5.93	± 0.40	7.10	± 0.71	6.56	± 0.91	5.77	± 0.73	5.11	± 0.40	5.57	± 0.37	5.51	± 0.74	5.67	± 0.59	6.17	± 0.27
No HCO_3	5.92	± 0.16	5.71	± 0.39	7.34	± 0.42	7.49	± 0.44	7.00	± 0.28	6.40	± 0.43	6.48	± 0.23	6.26	± 0.13	6.64	± 0.32	7.13	± 0.37
No saline	5.46	± 0.20	5.54	± 0.18	7.50	± 0.59	7.34	± 0.33	7.03	± 0.75	6.79	± 0.51	6.90	± 0.41	6.85	± 0.51	7.06	± 0.55	7.18	± 0.47
All therapy	5.62	± 0.17	5.29	± 0.26	6.70	± 0.32	7.13	± 0.33	6.40	± 0.20	6.25	± 0.15	6.06	± 0.34	5.52	± 0.54	5.89	± 0.46	6.36	± 0.65

Table I.58 Serum chemistry values ± SEM.

	Hours Postdosing									
	-1	0	1	2	3	4	5	6	7	8
Alanine Amino Transferase (U/L)										
No therapy	63.33 ± 6.64	63.00 ± 9.45	97.33 ± 25.64	94.67 ± 16.90	79.33 ± 9.06	98.67 ± 14.19	103.67 ± 12.02	107.67 ± 16.51	118.67 ± 21.84	130.33 ± 15.21
No A.C.	49.33 ± 4.33	53.33 ± 3.18	74.33 ± 6.17	62.00 ± 2.52	52.33 ± 2.73	48.67 ± 6.96	60.00 ± 2.08	60.33 ± 3.84	65.67 ± 1.20	114.33 ± 30.05
No HCO_3	62.00 ± 7.52	64.50 ± 3.28	88.75 ± 6.25	85.25 ± 8.84	69.75 ± 2.78	63.75 ± 2.78	69.25 ± 6.26	71.00 ± 11.85	70.50 ± 9.13	80.50 ± 11.15
No saline	58.33 ± 9.21	55.00 ± 6.11	86.67 ± 8.69	83.00 ± 6.08	66.00 ± 4.51	58.00 ± 2.08	59.67 ± 2.96	62.67 ± 5.90	74.00 ± 4.73	80.67 ± 4.33
All therapy	66.25 ± 13.22	72.00 ± 17.44	87.25 ± 11.69	75.00 ± 10.40	67.25 ± 12.17	65.25 ± 9.99	58.75 ± 10.61	59.25 ± 10.16	68.50 ± 8.70	89.00 ± 9.42
Albumin (gm/L)										
No therapy	2.90 ± 0.10	2.93 ± 0.12	3.00 ± 0.10	3.00 ± 0.06	2.77 ± 0.13	2.70 ± 0.15	2.60 ± 0.10	2.47 ± 0.19	2.50 ± 0.10	2.63 ± 0.15
No A.C.	2.70 ± 0.10	2.60 ± 0.10	2.60 ± 0.15	2.30 ± 0.20	1.90 ± 0.12	1.73 ± 0.13	1.73 ± 0.09	1.77 ± 0.09	1.67 ± 0.09	1.80 ± 0.10
No HCO_3	2.75 ± 0.12	2.72 ± 0.14	2.65 ± 0.18	2.47 ± 0.17	2.17 ± 0.13	1.90 ± 0.11	1.85 ± 0.10	1.82 ± 0.09	1.77 ± 0.09	1.80 ± 0.10
No saline	2.83 ± 0.13	2.70 ± 0.15	2.83 ± 0.13	2.83 ± 0.13	2.63 ± 0.13	2.40 ± 0.10	2.37 ± 0.07	2.30 ± 0.10	2.23 ± 0.09	2.27 ± 0.12
All therapy	2.70 ± 0.12	2.90 ± 0.23	2.88 ± 0.23	3.22 ± 0.57	2.30 ± 0.14	2.04 ± 0.13	1.90 ± 0.13	1.90 ± 0.15	2.06 ± 0.28	1.94 ± 0.20
Alkaline Phosphatase (U/L)										
No therapy	37.33 ± 2.19	37.00 ± 2.52	43.00 ± 4.04	46.33 ± 2.91	52.00 ± 2.00	63.00 ± 5.00	75.33 ± 8.69	8.33 ± 12.99	100.67 ± 18.53	126.33 ± 28.99
No A.C.	45.67 ± 5.55	44.33 ± 5.61	50.67 ± 5.55	54.00 ± 3.61	60.33 ± 1.20	66.67 ± 0.88	79.00 ± 2.89	73.67 ± 3.76	68.50 ± 0.50	69.33 ± 4.06
No HCO_3	45.75 ± 4.61	45.00 ± 5.51	52.50 ± 5.58	55.50 ± 3.52	70.75 ± 4.77	89.25 ± 13.28	117.25 ± 25.63	129.25 ± 37.19	138.75 ± 37.86	113.25 ± 31.27
No saline	44.67 ± 3.38	43.67 ± 3.38	50.00 ± 2.89	53.33 ± 2.67	57.00 ± 2.65	64.00 ± 1.53	78.67 ± 5.55	85.00 ± 7.77	83.00 ± 10.58	86.33 ± 8.95
All therapy	42.50 ± 1.55	42.75 ± 1.89	48.50 ± 1.71	51.50 ± 3.59	62.00 ± 12.73	72.50 ± 18.72	90.75 ± 28.52	98.75 ± 35.17	93.50 ± 27.52	121.50 ± 59.84
Aspartate Amino Transferase (U/L)										
No therapy	32.67 ± 2.03	33.67 ± 1.33	40.00 ± 3.61	43.67 ± 1.20	47.00 ± 3.61	50.00 ± 1.53	56.00 ± 2.08	63.67 ± 02.40	70.00 ± 4.36	93.33 ± 7.86
No A.C.	31.00 ± 0.58	28.00 ± 2.52	34.33 ± 2.85	47.67 ± 11.79	50.33 ± 9.61	52.67 ± 10.48	68.00 ± 13.08	79.33 ± 15.17	100.33 ± 26.86	139.00 ± 40.41
No HCO_3	26.75 ± 3.99	27.25 ± 3.68	34.00 ± 2.16	45.50 ± 4.57	51.50 ± 3.77	55.00 ± 4.24	60.50 ± 5.33	68.75 ± 09.75	75.00 ± 8.73	86.00 ± 10.21
No saline	30.00 ± 4.16	28.67 ± 5.67	35.00 ± 7.02	42.33 ± 12.00	50.33 ± 11.35	53.00 ± 13.32	58.67 ± 15.21	64.33 ± 17.68	71.33 ± 20.63	78.67 ± 19.19
All therapy	32.20 ± 4.20	35.60 ± 5.46	37.20 ± 6.65	50.60 ± 7.11	51.60 ± 11.28	66.00 ± 15.95	77.20 ± 18.01	89.60 ± 23.44	113.40 ± 29.68	133.60 ± 44.07
Blood Urea Nitrogen (mg/dl)										
No therapy	7.33 ± 2.19	6.67 ± 1.86	8.67 ± 1.45	11.00 ± 1.73	15.33 ± 1.45	19.33 ± 2.40	22.33 ± 2.85	30.33 ± 8.95	34.33 ± 9.40	41.67 ± 13.25
No A.C.	9.33 ± 0.33	8.33 ± 0.33	8.67 ± 0.67	10.00 ± 0.58	11.33 ± 0.67	13.33 ± 0.67	15.33 ± 0.88	18.00 ± 1.53	20.67 ± 1.45	24.67 ± 0.67
No HCO_3	7.50 ± 0.50	7.75 ± 0.48	7.50 ± 0.29	9.00 ± 0.71	10.25 ± 0.48	11.50 ± 0.65	12.25 ± 0.85	14.25 ± 1.11	15.25 ± 0.85	17.75 ± 1.49
No saline	8.33 ± 1.45	8.00 ± 1.73	9.00 ± 1.53	10.0 ± 1.2	14.00 ± 1.15	16.67 ± 1.45	19.33 ± 2.19	21.00 ± 3.06	24.67 ± 4.37	27.67 ± 4.37
All therapy	10.98 ± 3.59	10.88 ± 3.58	10.08 ± 2.10	11.82 ± 1.62	14.02 ± 2.33	15.18 ± 1.95	16.02 ± 0.92	16.98 ± 1.63	19.70 ± 1.92	21.40 ± 1.81

Table I.58 continued

	Hours Postdosing																	
	-1		0		1		2		3		4		5		6		7		8	
Chloride (mEq/l)																				
No therapy	99.00	± 1.15	98.67	± 2.96	97.00	± 8.00	107.67	± 1.20	108.33	± 3.28	110.67	± 2.03	113.33	± 1.86	113.00	± 1.00	113.33	± 0.88	114.67	± 0.88
No A.C.	102.33	± 1.45	103.00	± 1.15	109.67	± 2.40	114.67	± 0.67	117.00	± 2.08	119.00	± 1.73	120.33	± 0.33	120.33	± 1.86	121.67	± 3.48	122.33	± 3.71
No HCO_3	102.00	± 1.35	103.25	± 0.75	107.75	± 1.44	111.50	± 4.19	113.00	± 4.85	117.00	± 5.18	118.50	± 3.12	106.75	± 5.15	120.33	± 3.84	114.25	± 8.14
No saline	101.67	± 4.84	104.67	± 2.85	109.33	± 1.76	111.00	± 1.73	112.00	± 1.73	113.00	± 2.65	111.33	± 1.67	112.67	± 2.03	112.67	± 2.40	110.00	± 2.89
All therapy	98.20	± 4.16	104.00	± 0.63	104.40	± 4.42	110.60	± 1.63	107.00	± 7.04	110.80	± 8.01	112.60	± 7.02	113.20	± 9.94	115.40	± 5.95	115.80	± 8.42
Cholesterol (mg/dl)																				
No therapy	96.00	± 7.94	97.33	± 6.33	102.67	± 5.46	96.67	± 4.33	93.00	± 5.13	85.33	± 2.33	80.67	± 4.37	79.00	± 5.13	76.00	± 3.79	80.33	± 6.01
No A.C.	123.33	± 0.88	113.67	± 3.93	123.00	± 6.66	109.67	± 8.19	91.00	± 4.00	79.00	± 7.55	78.00	± 4.93	73.67	± 3.48	69.67	± 3.18	69.67	± 2.73
No HCO_3	130.00	± 9.81	121.00	± 10.46	117.00	± 4.74	103.75	± 4.23	96.25	± 3.71	88.00	± 3.72	82.00	± 5.37	81.25	± 7.28	78.00	± 6.89	78.75	± 7.17
No saline	137.00	± 11.15	130.33	± 9.91	132.67	± 10.68	133.00	± 10.97	123.33	± 7.88	108.67	± 11.57	102.33	± 11.57	87.67	± 19.88	87.00	± 20.30	82.33	± 19.34
All therapy	114.50	± 3.38	109.50	± 2.66	106.00	± 3.00	99.75	± 2.21	84.75	± 1.60	68.25	± 8.83	60.50	± 7.17	57.75	± 6.80	58.00	± 8.21	55.25	± 7.22
Creatinine (mg/dl)																				
No therapy	1.20	± 0.21	1.13	± 0.17	1.10	± 0.15	1.33	± 0.26	1.50	± 0.17	2.20	± 0.15	2.30	± 0.26	2.70	± 0.38	3.23	± 0.15	3.57	± 0.15
No A.C.	1.13	± 0.09	1.10	± 0.06	1.23	± 0.09	1.20	± 0.06	1.20	± 0.00	1.23	± 0.09	1.50	± 0.10	1.53	± 0.09	1.73	± 0.19	2.00	± 0.10
No HCO_3	1.20	± 0.07	1.17	± 0.05	1.27	± 0.08	1.30	± 0.09	1.25	± 0.10	1.35	± 0.09	1.45	± 0.12	1.60	± 0.15	1.77	± 0.17	1.85	± 0.22
No saline	0.97	± 0.15	0.93	± 0.18	1.07	± 0.20	1.13	± 0.23	1.40	± 0.10	1.53	± 0.17	1.60	± 0.20	1.67	± 0.24	1.77	± 0.30	2.13	± 0.19
All therapy	1.14	± 0.11	1.22	± 0.10	1.30	± 0.14	1.34	± 0.14	1.26	± 0.15	1.30	± 0.17	1.30	± 0.17	1.36	± 0.23	1.50	± 0.21	1.62	± 0.22
Globulin (gm/dl)																				
No therapy	2.50	± 0.40	2.57	± 0.37	2.63	± 0.30	2.67	± 0.27	2.33	± 0.19	2.30	± 0.25	2.17	± 0.22	2.20	± 0.35	2.10	± 0.25	2.20	± 0.26
No A.C.	2.37	± 0.38	2.20	± 0.29	2.30	± 0.38	2.03	± 0.24	1.53	± 0.07	1.37	± 0.03	1.40	± 0.06	1.27	± 0.07	1.33	± 0.03	1.30	± 0.10
No HCO_3	2.47	± 0.15	2.40	± 0.22	2.35	± 0.28	1.97	± 0.31	1.90	± 0.33	1.60	± 0.11	1.45	± 0.03	1.45	± 0.03	1.45	± 0.05	1.45	± 0.05
No saline	2.77	± 0.32	2.60	± 0.25	2.67	± 0.27	2.73	± 0.28	2.53	± 0.27	2.27	± 0.23	2.20	± 0.17	2.20	± 0.15	1.90	± 0.35	1.53	± 0.20
All therapy	2.65	± 0.10	2.20	± 0.21	1.90	± 0.25	2.02	± 0.27	1.48	± 0.24	1.38	± 0.23	1.22	± 0.19	1.16	± 0.25	1.26	± 0.18	1.20	± 0.19
Glucose (mg/dl)																				
No therapy	110.00	± 2.89	119.00	± 6.08	119.67	± 7.86	165.33	± 47.13	158.67	± 44.18	77.67	± 21.49	53.00	± 2.65	48.33	± 3.18	42.33	± 8.35	35.00	± 1.00
No A.C.	112.00	± 8.54	123.33	± 13.86	94.00	± 12.49	95.33	± 7.13	88.00	± 7.21	71.00	± 3.21	66.00	± 1.73	57.00	± 7.09	59.67	± 10.73	65.00	± 18.36
No HCO_3	98.50	± 3.71	106.50	± 4.05	108.75	± 11.60	95.00	± 6.12	92.25	± 6.66	83.50	± 16.07	67.75	± 12.78	82.50	± 23.83	63.25	± 6.20	62.50	± 1.94
No saline	111.67	± 11.89	108.00	± 4.04	84.33	± 9.84	95.00	± 3.51	101.00	± 3.61	86.67	± 5.36	65.00	± 6.24	83.67	± 24.66	69.33	± 6.74	63.33	± 0.88
All therapy	107.80	± 3.47	108.00	± 4.16	97.60	± 5.42	104.80	± 6.37	100.60	± 15.78	83.20	± 19.36	54.00	± 11.18	44.20	± 7.95	44.00	± 10.61	51.20	± 9.50

Table I.58 continued

	Hours Postdosing									
	-1	0	1	2	3	4	5	6	7	8
Ionized Calcium (mEq/l)										
No therapy	5.37 ± 0.38	5.87 ± 0.30	4.80 ± 0.78	5.03 ± 0.19	4.37 ± 0.26	4.40 ± 0.36	4.50 ± 0.17	4.10 ± 0.06	4.03 ± 0.43	4.17 ± 0.12
No A.C.	3.87 ± 1.13	3.73 ± 1.23	4.00 ± 1.32	4.20 ± 0.83	3.53 ± 1.08	3.47 ± 0.75	3.27 ± 0.64	2.97 ± 0.47	2.83 ± 0.50	2.73 ± 0.58
No HCO_3	5.37 ± 0.37	5.60 ± 0.83	5.05 ± 0.34	4.77 ± 0.40	4.70 ± 0.14	4.53 ± 0.07	4.72 ± 0.25	4.30 ± 0.31	3.80 ± 0.21	3.65 ± 0.25
No saline	4.30 ± 0.91	3.43 ± 0.92	5.33 ± 0.44	5.67 ± 0.98	3.30 ± 0.50	3.60 ± 0.86	3.30 ± 0.44	2.95 ± 0.25	3.03 ± 0.75	2.67 ± 0.37
All therapy	4.12 ± 0.24	4.22 ± 0.41	4.04 ± 0.30	4.10 ± 0.30	4.18 ± 0.18	3.22 ± 0.51	3.44 ± 0.46	3.05 ± 0.44	2.98 ± 0.31	3.36 ± 0.14
Lactate Dehydrogenase (U/L)										
No therapy	251.50 ± 66.50	286.50 ± 56.50	270.50 ± 85.50	313.00 ± 94.00	270.00 ± 63.00	275.00 ± 64.00	309.00 ± 84.00	313.50 ± 74.50	317.00 ± 32.00	402.50 ± 65.50
No A.C.	258.00 ± 39.23	333.30 ± 13.43	369.33 ± 59.17	370.33 ± 60.87	353.00 ± 71.39	360.00 ± 47.32	399.33 ± 51.93	552.33 ± 78.15	520.67 ±134.04	616.33 ±149.05
No HCO_3	281.75 ± 40.06	335.00 ± 12.67	307.25 ± 33.40	382.50 ± 77.66	335.25 ± 17.91	330.50 ± 44.64	419.50 ± 78.38	522.25 ± 82.04	579.25 ±159.17	575.25 ±101.70
No saline	297.33 ± 49.99	274.00 ± 39.46	307.00 ± 49.27	323.33 ± 46.59	330.67 ± 28.03	324.33 ± 38.83	375.33 ± 37.44	343.00 ± 31.19	385.00 ± 19.60	467.33 ± 76.70
All therapy	310.50 ± 29.36	317.00 ± 36.14	348.50 ± 48.44	326.25 ± 6.25	284.25 ± 36.28	296.00 ± 43.60	342.50 ± 42.03	355.50 ± 29.89	393.75 ± 11.60	444.00 ± 33.72
Magnesium (mEq/l)										
No therapy	19.03 ± 0.86	19.70 ± 1.25	20.73 ± 1.68	20.83 ± 1.41	21.53 ± 1.78	20.70 ± 1.31	22.20 ± 1.74	22.93 ± 1.23	23.93 ± 1.60	26.60 ± 2.81
No A.C.	19.27 ± 1.29	18.40 ± 0.35	18.20 ± 0.87	17.80 ± 1.31	16.93 ± 1.35	16.63 ± 1.53	17.57 ± 1.65	16.47 ± 1.37	16.63 ± 1.51	17.93 ± 1.23
No HCO_3	20.22 ± 1.60	19.87 ± 1.23	19.75 ± 0.91	20.42 ± 0.97	18.90 ± 0.46	19.52 ± 0.81	22.77 ± 1.71	24.87 ± 2.84	24.32 ± 1.75	24.75 ± 2.67
No saline	21.37 ± 0.66	21.07 ± 0.98	19.40 ± 0.87	20.73 ± 0.72	21.63 ± 0.41	20.17 ± 0.43	26.53 ± 2.05	27.97 ± 1.66	28.03 ± 3.12	27.87 ± 2.10
All therapy	19.38 ± 0.99	19.90 ± 0.80	19.22 ± 0.85	20.16 ± 0.70	19.20 ± 0.54	19.42 ± 0.88	22.24 ± 1.55	26.82 ± 4.04	27.02 ± 3.33	28.20 ± 2.93
Phosphorous (mEq/l)										
No therapy	7.77 ± 0.41	8.23 ± 0.96	7.10 ± 0.47	7.13 ± 0.44	8.53 ± 0.30	9.63 ± 0.47	10.23 ± 0.41	9.70 ± 0.70	11.60 ± 0.66	12.87 ± 1.26
No A.C.	8.17 ± 0.33	8.10 ± 0.00	6.93 ± 0.20	6.83 ± 0.86	7.40 ± 0.90	7.40 ± 0.71	8.43 ± 0.83	8.17 ± 0.64	9.27 ± 0.54	11.07 ± 0.92
No HCO_3	7.80 ± 0.35	7.77 ± 0.34	6.45 ± 0.18	6.35 ± 0.17	6.75 ± 0.06	6.97 ± 0.25	6.97 ± 0.16	7.65 ± 0.76	8.25 ± 0.61	9.00 ± 0.51
No saline	7.97 ± 0.19	7.83 ± 0.29	6.73 ± 0.20	7.13 ± 0.43	8.23 ± 0.56	9.63 ± 2.95	10.80 ± 3.07	10.57 ± 2.76	12.77 ± 2.54	13.00 ± 2.80
All therapy	7.28 ± 0.59	7.86 ± 0.36	6.56 ± 0.42	6.66 ± 0.40	6.30 ± 0.52	6.82 ± 0.51	6.78 ± 0.38	6.78 ± 0.45	7.70 ± 0.55	8.12 ± 0.55
Potassium (mEq/l)										
No therapy	3.73 ± 0.39	3.83 ± 0.39	4.23 ± 0.23	4.87 ± 0.37	4.93 ± 0.19	5.50 ± 0.40	5.53 ± 0.43	5.73 ± 0.19	5.70 ± 0.17	7.07 ± 0.82
No A.C.	3.87 ± 0.20	4.13 ± 0.03	4.43 ± 0.13	4.57 ± 0.13	4.50 ± 0.25	4.27 ± 0.34	4.20 ± 0.31	4.10 ± 0.31	4.00 ± 0.31	4.40 ± 0.17
No HCO_3	3.95 ± 0.10	3.80 ± 0.11	4.10 ± 0.15	4.25 ± 0.15	4.15 ± 0.26	4.45 ± 0.10	4.57 ± 0.17	5.05 ± 0.31	5.12 ± 0.11	5.17 ± 0.27
No saline	3.90 ± 0.10	3.80 ± 0.06	4.07 ± 0.15	4.50 ± 0.10	4.80 ± 0.23	4.67 ± 0.37	4.47 ± 0.68	4.40 ± 0.83	4.93 ± 0.63	4.60 ± 0.40
All therapy	3.64 ± 0.16	3.86 ± 0.12	3.88 ± 0.16	4.46 ± 0.10	4.32 ± 0.32	4.30 ± 0.28	4.00 ± 0.23	3.96 ± 0.30	4.04 ± 0.17	4.22 ± 0.34

Table I.58 continued

	Hours Postdosing									
	-1	0	1	2	3	4	5	6	7	8
Protein Bound Calcium (mEq/l)										
No therapy	3.63 ± 0.58	3.33 ± 0.30	3.70 ± 1.10	3.00 ± 0.49	3.17 ± 0.47	2.70 ± 0.32	1.97 ± 0.12	2.13 ± 0.29	2.10 ± 0.35	2.13 ± 0.47
No A.C.	5.55 ± 1.25	5.43 ± 1.19	5.03 ± 1.30	3.67 ± 0.86	2.83 ± 0.69	2.53 ± 0.74	2.37 ± 0.62	2.27 ± 0.47	1.80 ± 0.23	1.93 ± 0.19
No HCO_3	3.90 ± 0.51	3.75 ± 0.78	3.82 ± 0.58	3.05 ± 0.72	2.30 ± 0.19	2.17 ± 0.18	1.57 ± 0.44	2.07 ± 0.15	2.50 ± 0.00	2.35 ± 0.09
No saline	5.17 ± 0.92	5.80 ± 1.07	3.73 ± 0.46	2.67 ± 1.03	4.45 ± 1.05	2.83 ± 0.82	2.77 ± 0.43	2.80 ± 0.30	2.27 ± 0.92	2.13 ± 0.54
All therapy	4.84 ± 0.41	4.48 ± 0.44	4.72 ± 0.46	4.18 ± 0.34	2.92 ± 0.67	3.14 ± 0.75	2.60 ± 0.92	3.50 ± 1.05	2.70 ± 0.81	2.24 ± 0.90
Sodium (mEq/l)										
No therapy	138.67 ± 0.33	140.67 ± 1.45	141.00 ± 1.73	142.00 ± 1.15	141.33 ± 1.86	143.33 ± 3.28	141.67 ± 2.03	142.00 ± 2.08	143.67 ± 1.20	145.33 ± 0.88
No A.C.	143.00 ± 1.53	143.00 ± 1.73	144.67 ± 0.67	147 00 ± 1.00	148.67 ± 0.88	150.00 ± 0.58	152.33 ± 0.67	153.67 ± 2.19	154.67 ± 2.19	159.00 ± 3.21
No HCO_3	142.50 ± 1.55	143.75 ± 0.63	144.50 ± 0.65	145.00 ± 1.08	139.75 ± 5.95	144.50 ± 0.96	144.50 ± 0.87	147.00 ± 1.96	146.25 ± 1.80	146.00 ± 1.58
No saline	143.00 ± 0.85	144.33 ± 0.88	145.00 ± 0.58	146.33 ± 0.88	147.00 ± 0.58	147.00 ± 1.53	148.00 ± 2.00	140.67 ± 7.31	150.33 ± 2.03	150.67 ± 1.76
All therapy	138.40 ± 5.42	143.80 ± 0.97	141.40 ± 4.89	145.40 ± 1.29	138.20 ± 8.07	139.60 ± 9.45	141.40 ± 8.16	139.60 ± 10.94	147.00 ± 6.98	146.40 ± 8.61
Total Calcium (mEq/l)										
No therapy	9.00 ± 0.25	9.20 ± 0.17	8.50 ± 0.40	8.03 ± 0.32	7.53 ± 0.26	7.10 ± 0.31	6.47 ± 0.27	6.23 ± 0.32	6.13 ± 0.28	6.30 ± 0.59
No A.C.	9.37 ± 0.15	9.17 ± 0.07	9.03 ± 0.03	7.87 ± 0.03	6.70 ± 0.10	6.00 ± 0.29	5.63 ± 0.20	5.23 ± 0.18	4.63 ± 0.39	4.67 ± 0.44
No HCO_3	9.27 ± 0.15	9.35 ± 0.17	8.87 ± 0.27	7.82 ± 0.34	7.00 ± 0.10	6.32 ± 0.38	6.30 ± 0.25	6.02 ± 0.36	5.70 ± 0.62	6.00 ± 0.20
No saline	9.47 ± 0.09	9.23 ± 0.18	9.07 ± 0.15	8.33 ± 0.15	7.70 ± 0.32	6.43 ± 0.12	6.07 ± 0.07	5.67 ± 0.09	5.30 ± 0.17	4.80 ± 0.44
All therapy	8.96 ± 0.54	8.70 ± 0.62	8.76 ± 0.47	8.28 ± 0.26	7.10 ± 0.75	6.36 ± 0.91	6.04 ± 0.94	5.90 ± 1.07	5.68 ± 0.88	5.60 ± 0.91
Total Protein (gm/l)										
No therapy	5.40 ± 0.30	5.50 ± 0.25	5.63 ± 0.20	5.67 ± 0.22	5.10 ± 0.06	5.00 ± 0.10	4.77 ± 0.12	4.67 ± 0.18	4.60 ± 0.15	4.83 ± 0.22
No A.C.	5.07 ± 0.46	4.80 ± 0.38	4.90 ± 0.53	4.33 ± 0.44	3.43 ± 0.18	3.10 ± 0.15	3.13 ± 0.12	3.03 ± 0.12	3.00 ± 0.10	3.10 ± 0.17
No HCO_3	5.22 ± 0.26	5.12 ± 0.35	5.00 ± 0.45	4.45 ± 0.40	4.07 ± 0.38	3.50 ± 0.13	3.30 ± 0.11	3.27 ± 0.11	3.22 ± 0.11	3.25 ± 0.13
No saline	5.60 ± 0.26	5.30 ± 0.23	5.50 ± 0.23	5.57 ± 0.26	5.17 ± 0.35	4.67 ± 0.24	4.57 ± 0.19	4.50 ± 0.15	4.13 ± 0.26	3.80 ± 0.15
All therapy	5.04 ± 0 35	5.10 ± 0.19	4.78 ± 0.39	4.84 ± 0.18	3.78 ± 0.27	3.42 ± 0.25	3.12 ± 0.18	3.06 ± 0.24	3.32 ± 0.30	3.14 ± 0.20

II. FATE AND DETECTION OF T-2 TOXIN AND ITS METABOLITES AS INFLUENCED BY DOSAGE ROUTE OF ADMINISTRATION, SPECIES, AND THERAPEUTIC INTERVENTION

ABBREVIATIONS USED

8-Ac TOL	8-acetoxy tetraol (TMR-2)
CHO	Chinese hamster ovary cells
CI	chemical ionization
4-DN	4-deacetylneosolaniol(15-acetoxy tetraol;TMR-1)
15-DN	4-acetoxy tetraol (N1)
DE MAS	deepoxy 15-monoacetoxyscirpenol
DE STRIOL	deepoxy scirpentriol
DAS	diacetoxyscirpenol
DE HT-2	deepoxy HT-2
DE TOL	deepoxy T-2 tetraol
DE TRIOL	deepoxy T-2 triol
DON	deoxynivalenol (vomitoxin)
DOM-1	deepoxy deoxynivalenol
FUS	fusarenone
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
HPLC	high performance liquid chromatography
ISO TC-1	3-acetyl-3'-hydroxy HT-2
NEO	neosolaniol
NIV	nivalenol
MAS	15-monoacetoxyscirpenol
4-MAS	4-monoacetoxyscirpenol
3'OH T-2	3'-hydroxy T-2 toxin (TB-1;TC-1)
3'OH HT-2	3'-hydroxy HT-2 (TC-3;TB-3)
3'OH TRIOL	3'-hydroxy T-2 triol
PB	phenobarbital
S-9	9,000 x g supernatant fraction
STRIOL	scirpentriol
TRIOL	T-2 triol
TOL	T-2 tetraol
TLC	thin-layer chromatography
TFA	trifluoroacyl
TMS	trimethylsilyl
TS	trichothecene skeleton
VERO	African green monkey kidney cells

A. Literature Review: The Distribution, Metabolism, and Excretion of Trichothecene Mycotoxins

1. Chemical and physical properties of trichothecenes

All trichothecene mycotoxins have a basic tetracyclic sesquiterpene structure with a six-membered oxygen-containing ring, an epoxide group in the 12,13 position and an olefinic bond in the 9,10 position. The trichothecenes may also have side groups such as hydroxyl, esterified hydroxyl, keto, or epoxide groups in various combinations (Figure II.1). Ueno (1980) has classified the trichothecenes into four basic groups according to their structural characteristics. Group A trichothecenes possess hydroxyl or esterified hydroxyls at the 3, 4, 7, 8, or 15 positions. Group B trichothecenes contain a carbonyl group at the C-8 position in addition to other functional groups in group A. Group C trichothecenes are characterized by a second epoxide at the 7,8 position while group D comprises the macrocyclic trichothecenes which bear a bridge of varying length and composition between carbons C-4 and C-5.

Diacetoxyscirpenol (DAS), T-2 toxin, deoxynivalenol (DON, vomitoxin), and nivalenol (NIV) are four of the more important trichothecene mycotoxins (Figure II.1). All have been detected in naturally contaminated feeds or foods. Of these four toxins, T-2 has been studied to the greatest extent and until recently, information was largely limited to this compound.

2. General xenobiotic metabolism

There are four basic mechanisms for the biotransformation of xenobiotics commonly employed by animals. These include oxidation, reduction, hydrolysis, and synthesis or conjugation. The first three are commonly referred to as Phase I reactions and are frequently only the first stages of biotransformation. The products of metabolism need not be less toxic. In some instances, the products may be more toxic (bioactivation) or of equal toxicity to the parent compound. Synthesis or conjugation reactions are Phase II reactions which usually act to greatly increase the water solubility of the compound and, in general, abolish biological activity.

Significant species variations in xenobiotic metabolism have been documented. Major differences may result from a particular mode of biotransformation defective or an especially well developed by a given species. For example, cats are generally deficient in their ability to form glucuronides but may compensate to a degree by forming sulfate conjugates (Gregus et al., 1983; Testa and Jenner, 1976; Capel and Williams, 1974). In contrast, swine are deficient in forming sulfates but readily form glucuronides (Testa and Jenner, 1976; Capel and Williams, 1974). Other species differences arise from differences in enzyme concentrations, enzyme specificities, the presence of enzyme inhibitors, enhanced enzymatic activity for reverse reactions, and/or the nature and extent of competitive pathways.

As for the metabolism of trichothecenes, all four basic reactions have been reported. These include hydrolysis (of ester linkages), oxidation (aliphatic hydroxylation of the C-3' or C-7 carbon of T-2), reduction (of the 12,13-epoxide to yield a carbon-carbon double bond), and synthesis (glucuronide conjugation, acetylation). While hydrolysis, oxidation, and synthesis of trichothecenes occur in the body of animals, the reduction

of the 12,13-epoxide is likely to occur primarily through the action of microorganisms in the anaerobic environment of the gastrointestinal tract. Ruminants or species with a well-developed cecum should be particularly adept at this reaction. The fourth type of reaction, conjugation, has only recently been reported for trichothecenes but may account for many very polar metabolites that were reported but not structurally identified in several earlier animal studies.

3. In vitro metabolism of trichothecenes

The ability of animal livers to metabolize T-2 toxin was initially reported by Ellison and Kotsonis (1974). They detected HT-2 as the sole metabolite in in vitro studies with human and bovine liver S-9 (9,000 x g liver supernatant fraction) homogenates. The half-life of T-2 in this system was 20 minutes in human liver compared to 40 minutes in bovine liver, suggesting a faster rate of metabolism in humans. HT-2 was not detected upon incubation of T-2 toxin with human plasma or treatment with simulated gastric juice indicating simple chemical hydrolysis of T-2 to HT-2 was not an important reaction.

HT-2 was also the sole metabolite of T-2 toxin after incubation with microsomal fractions from a variety of animals and organs (Ohta et al., 1977). Esterase activity which selectively hydrolyzed the C-4 acetyl group in T-2 toxin to yield HT-2 was found mainly in the microsomal fraction of the liver, kidney, and spleen of laboratory animals. Very little if any activity was detected in microsomes prepared from brain, intestines, or in blood serum or blood cells. The microsomal fraction from rabbit livers was significantly more active in deacylating T-2 toxin when compared to human, mouse, chicken, rat, or guinea pig liver microsomes. Enzymatic deacylation activity was inhibited by the organophosphorus compounds eserine and diisopropylfluorophosphate, known inhibitors of esterases. Since no T-2 was converted to HT-2 by blood cells or serum, the authors concluded that nonspecific carboxyesterases catalyzed the deacylation of T-2 toxin to yield HT-2.

The substrate specificities of rat liver microsomal nonspecific carboxyesterases were subsequently examined using six type B and seven type A trichothecenes (Ohta et al., 1978). Trichothecenes containing a C-4 acetyl group (DAS, T-2, fusarenon-X, diacetylnivalenol) were selectively hydrolyzed to yield the C-4 deacylated products monoacetoxyscirpenol (MAS), HT-2, nivalenol and 15-acetyl nivalenol, respectively. Deacylation also occurred at the C-3 position of 3-acetyldeoxynivalenol and the C-8 position of tetraacetoxyscirpenol. Neosolaniol, HT-2, acetyl T-2 and tetraacetylnivalenol did not react under these conditions. In all cases, acetyl groups at the C-15 position resisted attack by hepatic microsomal esterases. The authors (Ohta et al., 1978) concluded hepatic microsomal esterases selectively attacked the C-4 acetyl group of trichothecenes but substituents at the C-3 and C-8 position decreased enzymatic activity.

In early studies of the metabolism of T-2 toxin by rat liver homogenates, ethyl acetate or chloroform were used to extract metabolites from the aqueous phase (Ohta et al., 1977; Ellison and Kotsonis, 1974). In later studies, columns packed with Amberlite XAD resins were utilized to extract toxin residues. Such columns were able to extract polar metabolites which were not readily partitioned from aqueous homogenates with ethyl acetate or chloroform. Yoshizawa et al. (1980b) examined the metabolism of T-2 toxin by rat liver S-9 (9,000 x g supernatant fraction)

homogenates utilizing Amberlite XAD columns for extraction of the homogenates. The rat liver S-9 homogenates converted T-2 toxin into the products HT-2, T-2 tetraol, 4-deacetylneosolaniol (4-DN), and trace amounts of an unknown designated TMR-2 (see Figure 1-2 for structures). The same metabolites were obtained with HT-2 as a substrate and it was concluded T-2 was preferentially hydrolyzed at the C-4 position to yield HT-2, which was then biotransformed to T-2 tetraol via 4-DN. In the same study, HT-2 and small amounts of neosolaniol were also detected upon incubation of T-2 toxin with rat intestinal and stomach strips; however, the more polar metabolites 4-DN and T-2 tetraol were not produced under these conditions.

Upon incubation of T-2 toxin with monolayer cell cultures of rat hepatocytes, T-2 toxin was rapidly converted to HT-2 (O'Brien et al., 1985). By comparison, HT-2 was only slowly metabolized by this cell type to yield several unidentified metabolites. T-2 tetraol was resistant to metabolism rat hepatocytes in this system. In contrast to hepatocytes, T-2 toxin was resistant to metabolism by VERO cells.

The metabolism of tritium labeled T-2 toxin was also studied in Chinese hamster ovary (CHO) cells, African Green monkey kidney (VERO) cells, human fibroblasts, and mouse conective tissue cells (Trusal, 1986). CHO and VERO cells metabolized T-2 to a much greater extent than either human fibroblast or mouse connective tissue cells. The major metabolite in CHO cells was HT-2, whereas in VERO cells the major metabolite was an unknown, more polar than T-2 by normal phase silica TLC. Smaller amounts of T-2 triol, T-2 tetraol and several additional unknowns were detected in the cell and media extracts of both CHO and VERO cells. In both cell lines metabolites were detected by 1 hour and after 4 hours only 37 to 58% of the added T-2 toxin remained intact. Although the major metabolite produced in VERO cells was not characterized, it is most likely 3'-hydroxy T-2, judging from the TLC profiles (i.e., R_f of the unknown was between T-2 and HT-2).

Data presented in these studies illustrate differences not only in the metabolizing activity but also in the specific enzymatic pathways for biotransformation of trichothecenes among different animal tissues and cell types. Although trichothecenes containing a C-4 ester functional group are rapidly hydrolyzed by esterase activity, other esters and especially carbon atoms in the basic trichothecene skeleton are more resistant to reaction.

Fronnum et al. (1985) separated and purified carboxylesterases obtained from rat liver microsomes by a variety of techniques including gel filtration, affinity chromatography, isoelectric focusing, and chromatofocusing. Five isoenzymes were identified which differed in molecular weight, isoelectric points, and substrate and inhibitor affinities. Only one of the microsomal carboxylesterase isoenzymes, with a pI of 5.4, metabolized T-2 and HT-2 was the sole metabolite detected. In contrast, 4-DN together with HT-2 were produced upon incubation of T-2 with the high-speed supernatant (S-9) fraction from rat liver homogenates. The microsomal carboxyesterase metabolism of T-2 to HT-2 was inhibited by paraoxon but unaffected by EDTA or 4-hydroxy mercury benzoate, inhibitors of aryl esterases, and esterases with thiol funtional groups in their active sites (Johnsen et al., 1987). These studies clearly delineated the high degree of specificity displayed by different carboxylesterases present in microsomal and cytoplasmic fractions.

Although both in vitro and in vivo studies in several animal species have demonstrated that T-2 is rapidly hydrolyzed to HT-2 by esterases, the in vitro metabolic oxidation of T-2 toxin was not reported until much later (Yoshizawa et al., 1984). Both T-2 toxin and HT-2 were hydroxylated at the C-3' position to yield 3'-hydroxy T-2 and 3'-hydroxy HT-2, respectively, by mouse or monkey liver homogenates supplemented with a NADPH generating system (see Figure II.2 for structures). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen are required for oxidation of xenobiotics by cytochrome P450 (mixed function oxidase) enzyme systems (Sipes and Gandolfi, 1986). T-2 toxin hydroxylation activity was present only in microsomal fractions supplemented with NADPH; no activity was observed in the cytosol. Hydroxylation activity was enhanced by pretreatment of the animals with phenobarbital. These findings suggested the reaction was catalyzed by cytochrome P450 mixed function oxidases. Hydrolysis products of T-2 including HT-2, neosolaniol, 4-deacetylneosolaniol (4-DN), 15-deacetylneosolaniol (15-DN), and T-2 tetraol were also detected. Analysis of reaction products over time revealed that 3'-hydroxy HT-2 and T-2 tetraol were resistant to further biotransformation indicating that they may be end products resistant to further metabolism.

Conversion of 3'-hydroxy HT-2 to neosolaniol, 4-DN, 15-DN, or T-2 tetraol did not occur demonstrating that C-3' hydroxylation inhibits hydrolysis of C8 esters by esterase enzymes. Such inhibition most likely accounts for the buildup of 3'-hydroxy HT-2 as a major metabolite of T-2 in vivo.

Phenylmethylsulfonyl fluoride, eserine sulfate, diisopropylfluorophosphate and diethyl p-nitrophenyl phosphate (paraoxon), all well known esterase inhibitors, blocked the deacylation of the C-4 acetyl group of T-2 toxin by pig liver S-9 fractions, although paroxon was the most potent inhibitor (Wei and Chu, 1985). Addition of paroxon to liver S-9 fractions obtained from phenobarbital pretreated swine supplemented with a NADPH generating system not only inhibited ester hydrolysis, but also shifted metabolism to favor oxidation of the C-3' carbon producing 3'-hydroxy T-2 toxin as the predominant product. In a similar manner, the addition of acetyl T-2, HT-2, and T-2 triol yielded their corresponding 3'-hydroxy derivatives demonstrating that enzymatic oxidation of the isovaleryl group is not restricted solely to T-2 toxin.

The metabolic profiles of T-2 toxin incubated with phenobarbital (PB) pretreated and control rat liver microsomes were studied by Knupp et al., (1986). The major metabolite produced by both treatment groups over incubation times from 5 to 60 minutes was HT-2, confirming that enzymatic ester hydrolysis at the C-4 position occurs more rapidly than hydroxylation at C-3' or ester hydrolysis at carbon atoms C-15 or C-8. In all, six identifiable metabolites including HT-2, 3'OH T-2, 3'OH-HT-2, neosolaniol, T-2 triol and 4-DN were produced within 5 minutes incubation by the PB-induced microsomes.

Treatment of rats with PB induced both esterase and mixed function oxidase activity, although the latter was increased to a much greater extent. The ratio of substrate to microsomal protein was critical in both the extent and pathway of metabolism. When the T-2/protein ratio was dropped from 975 nmoles/mg protein to 39 nmoles/mg protein the percentage of 3'-hydroxy T-2 formed increased three-fold, with no corresponding change in the percentage of HT-2 produced. This suggests that T-2 would be more extensively metabolized via oxidation at the C-3'

position in animals given lower doses of toxin while the formation of HT-2 would be more predominant in animals exposed to higher toxin doses.

The metabolism of T-2 toxin by hepatic microsomes prepared from phenobarbital-induced and control rats, chickens, mice, and rabbits was compared by Knupp et al. (1987a). The major metabolite in microsomal preparations from both control and PB induced rats, mice, and rabbits was HT-2. In microsomes prepared from PB induced chickens, 3'-hydroxy T-2 was the major metabolite, however, 30% and 79% of the added T-2 remained umetabolized in PB-induced and control chicken microsomal preparations. In all four species, hydroxylation products were increased over controls following PB treatment. Paraoxon added to the incubation mixtures significantly inhibited ester hydrolysis and resulted in a 1.4- and 1.25-fold increase in the percentage of 3'-hydroxy T-2 detected in the mice and rat microsomal samples, respectively. In the rabbit, 3'-hydroxy T-2 was not detected in the absence of paraoxon.

Two new metabolites of T-2, designated RLM-2 and RLM-3, were detected in chicken, rat, and mouse microsomal incubation mixtures. The metabolite RLM-3 was subsequently identified as 4'-hydroxy T-2 by GC-MS and ^{1}H and ^{13}C NMR (Knupp et al., 1987b). Rat skin irritation bioassays demonstrated that the new metabolite, 4'-hydroxy T-2, was nearly equal in dermal toxicity to that of T-2 toxin, indicating oxidation at the C-4' position is not a significant detoxification reaction.

Initial studies on the <u>in vitro</u> metabolism of trichothecenes were limited to the assessment of biotransformation by animal tissues or organs. These limitations, however, do not take into account the fact that in animals orally exposed to toxins, the compounds would be exposed to microorganisms present in the rumen and/or gastrointestinal tract prior to systemic absorption. Over 400 species of bacteria have been identified in the intestinal tract, and it has been suggested that gastrointestinal microflora have the potential capacity for the biotransformation of xenobiotics equal or greater to that of the liver (Sipes and Gandolfi, 1986).

The capacity of protozoa or bacteria in intact rumen fluid to metabolize several mycotoxins was first investigated by Kiessling et al. (1984). Rumen fluid or their microflora had no effect on DON, however, T-2 was converted to HT-2 and DAS to 15-monoacetoxyscirpenol (MAS). In a subsequent study, King et al. (1984) found that the epoxide group of DON was reduced to a carbon-carbon double bond (Figure II.2b) by bovine rumen microorganisms under anaerobic conditions to yield a deepoxy product. The metabolite was identical to the deepoxy DON metabolite labeled DOM-1, isolated during <u>in vivo</u> experiments using rats (Yoshizawa et al., 1983).

Deepoxy DON (DOM-1) was also detected upon anaerobic incubation of DON with bovine rumen microorganisms in our laboratory (Swanson et al., 1986a). In addition, DAS and T-2 toxin were reduced in a similar manner to yield deepoxy products. Deepoxy T-2 and deepoxy DAS were, however, not detected directly. Instead, T-2 and DAS were hydrolyzed at the C-4 position and the deacylated products were converted to their corresponding deepoxy products including deepoxy HT-2, deepoxy T-2 triol, deepoxy MAS, and deepoxy scirpentriol, respectively. With bovine rumen microorganisms, the deepoxy metabolites of both T-2 and DAS were significant products after 48 hours' incubation, but the simple hydrolysis products were predominant indicating that deacylation of the C-4 acetyl group by

microbial esterases occurred prior to the deepoxidation reaction. The proposed pathway for the metabolism of T-2, DAS, and DON by anaerobic microorganisms (given in Figure II.4) illustrates both enzymatic reduction and hydrolysis reactions.

Munger et al. (1987) also investigated the metabolism of T-2 toxin by bovine rumen fluid. They found no evidence for the reduction of the epoxide group to a diene by rumen microflora. However, both acetylation and deacetylation reactions were observed. Products of their incubations included acetyl T-2, HT-2, and acetyl HT-2. Additional experiments where di-isopropyl fluorophosphate was added to inhibit ester hydrolysis revealed that both acetylation and deacetylation reactions occur preferentially at the C-3 position compared to the C-4 position. The authors suggested acetylation of T-2 increases lipophilicity and may result in enhanced absorption and a corresponding increase in toxicity due to greater bioavailability of the more lipophilic metabolites.

Significant discrepancies between results obtained from various investigators upon incubation of trichothecenes with bovine rumen microorganisms have been noted. Three important factors which may account for these discrepancies in metabolites produced are: 1) concentration of toxin in the incubation mixture, 2) total incubation time, 3) the specific trichothecene investigated, and 4) oxygen tension. Munger et al. (1987) reported that toxin concentration was an important factor in the acetylation reactions with T-2 toxin; at concentrations above 10 ppm T-2, no acetylation products were observed, only the deacylated product HT-2. Neither Kiessling et al. (1984) nor Munger et al. (1987) detected any epoxide reduction products, however both groups used relatively short incubation times compared to the longer incubation times reported by King et al. (1984). Only with the latter group was epoxide reduction observed. In addition, only King et al. (1984) reported using rigorous techniques to exclude oxygen from their incubation systems. As the organsims responsible for deepoxidation appear to be anaerobes, incubation time, and oxygen tension should be related. Incubation time could be even more critical if the initial amount of oxygen present in the incubation system was too high and resulted in inhibition of the essential anaerobic microflora. In such a situation, only with longer incubation times would a reducing atmosphere be established.

Mixed flora from murine intestinal contents reduced T-2 toxin to deepoxy products (Yoshizawa et al., 1985a). The microorganisms involved in this reaction were shown to be anaerobes since the deepoxidation reaction did not occur under aerobic conditions. In addition to T-2 toxin, incubation of HT-2, NEO, 3'T-2, and 3'HT-2 with suspensions of murine intestinal microflora also yielded deepoxy products. Deepoxy products of T-2, 3'OH T-2, and NEO, compounds with C-4 esters, were not directly detected but rather their C-4 deacylated products, DE HT-2, DE 3'OH HT-2, and DE 4-DN, respectively. The authors concluded enzymatic deepoxidation was sterically inhibited by the presence of an ester functional group at the C-4 position, and deoxygenation occurred as a direct one step enzymatic reduction of the epoxide ring. However, several researchers have shown that hydrolysis of the C-4 ester group in T-2 and related trichothecenes occurs very rapidly within minutes, whereas epoxide reduction reactions have not been observed to occur until many hours or even days of incubation time. It, therefore, appears unlikely that C-4 hydrolysis is a limiting step in epoxide reduction.

The 12,13-epoxide group of trichothecenes is remarkably resistant to reaction. Most naturally occurring epoxides and epoxides in general are very reactive. Epoxide groups are typically detoxified in animals by several enzyme systems including: glutathione-S-transferase, epoxide hydratase, and/or epoxide reductase. Glutathione-S-transferase catalyzes the addition of glutathione to the epoxide group and other electrophiles. Epoxide hydratase is closely associated with cytochrome P-450 within the endoplasmic reticulum of animals and catalyzes the conversion of epoxide to transdihydrodiols. The trichothecenes are resistant to reaction with these enzyme systems. They are not substrates in vitro, for either glutathione-S-transferase, epoxide hydrolase, or vitamin K_1 epoxide reductase obtained from rat liver preparations (Bieger and Dose, 1985).

Documentation for the in vitro conjugation of trichothecenes was the last of the four major biotransformation reactions to be reported. The glucuronide conjugate of 15-MAS was the major product after incubation of DAS with uridine diphosphoglucuronic acid (UDPGA) and liver microsomes from ß-naphthoflavone-induced rats (Roush et al., 1985a). Similar results were obtained with T-2 toxin under these conditions; i.e., incubation of T-2 with microsomes yielded the glucuronide of HT-2 (Roush et al., 1985b). Synthesis of the metabolites and subsequent NMR studies revealed that the sugar moiety was attached in a @ glycoside linkage at the C-3 position with both MAS and HT-2. There was no evidence for the direct conjugation of either parent toxin, DAS or T-2, in these studies. This data suggests that trichothecenes with a C-4 ester such as DAS and T-2, are rapidly hydrolyzed at the C-4 position to yield MAS and HT-2, which are subsequently conjugated. It is not presently known whether the C-4 acetyl group inhibits the enzymatic glucuronidation of DAS or T-2 or whether the C-4 esters are hydrolyzed so rapidly under the conditions investigated that direct conjugation with glucuronic acid could not occur. The later is the most likely hypothesis since glucuronide conjugates of T-2 toxin have been detected in plasma and bile of swine administered T-2 toxin (Corley et al., 1985).

Using isolated perfused rat livers, Pace et al. (1986) studied the metabolism and clearance of [^{3}H] T-2 toxin. The rate of residue excretion (total radioactivity) into the bile was constant after 10 minutes initial perfusion. The toxin was delivered at a rate of 33.9 µg T-2/minute. Over 93% of the delivered mycotoxin was extracted and biotransformed by the liver. Only 4.6% of the added toxin remained as parent compound in the effluent perfusate. The major metabolites (% of total metabolites residues) detected in the bile were glucuronide conjugates (88%), 3'-hydroxy HT-2 (4%), 3'-hydroxy triol (1.5%), T-2 tetraol (3%), HT-2 (1%), 4-deacetylneosloaniol (1%), T-2 triol (0.5%), 3'-hydroxy T-2 (0.2%), and parent T-2 (0.3%). The polar conjugates were primarily glucuronide conjugates of HT-2 (80%), with smaller quantities of 3'-hydroxy HT-2 (11%) and T-2 tetraol (1%). Of particular interest was the observation that the glucuronide conjugates were poor substrates for bovine ß-glucuronidase. In contrast, ß-glucuronidase from limpets hydrolyzed greater than 92% of the polar conjugates remaining at the origin of TLC plates (developed in a two-phase system composed of chloroform-ethyl acetate-ethanol in ratios of: 1) 50+25+25 and 2) 80+10+10). However, the pH of the buffer systems utilized were not reported. Since the pH optima for ß-glucuronidase varies greatly between sources of the enzyme, pH of the buffer system employed can be a critical factor in the results obtained. The pH optima for ß-glucuronidase from limpets, E. coli and bovine liver sources are 3.8, 6.8, and 5.0, respectively.

Additional trichothecene metabolism studies using isolated perfused rat livers were investigated by Gareiss et al. (1986). The authors perfused isolated rat livers with 2 mg of either T-2 or DAS using a recirculating perfusion apparatus. Bile was extracted with ethyl acetate, with and without incubation with E. coli ß-glucuronidase, followed by GC-MS analysis of the TFA derivatives. The major metabolite detected in bile of livers perfused with T-2 toxin was the glucuronide conjugate of HT-2. In addition to small amounts of HT-2, two other metabolites were detected by GC-MS, 3'-hydroxy HT-2 and 3'-hydroxy-7-hydroxy HT-2. Quantitation of the latter two metabolites was not reported as analytical standards were not available to the investigators and identification was based upon published spectra (Visconti et al., 1985a,b).

In contrast to T-2 toxin, only two metabolites were found in the bile obtained from isolated rat livers perfused with DAS. The major compound was the glucuronide of MAS, although minor amounts of scirpentriol glucuronide were also found. No parent DAS or other free metabolites were detected.

In contrast to T-2 toxin or DAS, conjugates of DON were not formed upon incubation of DON with rat or swine hepatic microsomal preparations (Côté et al., 1987). No new chromatographic peaks were observed upon incubation of DON with the microsomes, nor was there any noticably loss of added parent compound. Similar results were obtained upon incubation of DOM-1 with the rat liver microsomal system. In order to determine if there were significant differences in metabolism of DON in vitro and in vivo, three rats and one pig were orally dosed with 2 mg/kg DON. As with the microsomal incubations, no glucuronide conjugates were detected in the urine of these animals. The lack of microsomal conjugation with DON by rats and swine, either in vivo or in vitro, is in contrast to results with other trichothecenes such as T-2 or DAS. Although the reason for this disparity has not been established, it is most likely due to the greater polarity and water solubility of DON.

4. Whole animal metabolism, distribution, and excretion of trichothecenes

a. Fusarenon-X

Studies on the disposition of trichothecene mycotoxins at the whole-animal level were reported by Japanese researchers as early as 1971. Ueno et al. (1971) first reported on the distribution and excretion of tritium-labeled fusarenon-X, a toxic trichothecene mycotoxin isolated from cultures identified as F. nivale Fn2B and F. epishaeria FnM. Tritium-labeled fusarenon-X prepared by the tritium gas exchange method (Wilzbach, 1957) was administered subcutaneously to four male mice at a dose of 4 mg/kg b.w. (3.6×10^5 cpm/animal, specific activity 0.9 mCi/mol). Maximum tissue radioactivity was reached in the first 30 minutes followed by a rapid decrease. Liver contained the greatest amount of radioactivity (2.4% of the dose) followed by kidney (1.0%) small intestine (0.8%) and large intestine (0.6%). By 12 hours, however, no radioactivity was detected in any tissue. This rapid decrease of radioactivity in tissues was matched by an increase of radioactivity in the excreta, with most detected in the urine. Radioactivity was extracted from urine with activated charcoal and subsequently eluted with methanol prior to chromatographic analysis. Negligible amounts of the parent fusarenon-X were detected in urine extracts by thin-layer chromatography. The majority of radioactivity

extracted from urine remained at the origin of the TLC plates indicating that fusarenon-X was metabolized to (a) more polar compound(s).

The total percentages of the administered dose recovered in this study ranged from only 6 to 24%. These low recoveries may be attributable to lability of the tritium labels (stability of the radiolabel was not reported), the extremely low specific activity of the compound, and/or the fact that only selected tissues were analyzed. The study, nevertheless, demonstrated that mice can rapidly metabolize and eliminate fusarenon-X, primarily via the urine, following subcutaneous administration. In later experiments, nivalenol was detected in the feces and urine of animals given fusarenon-X (Ueno, 1977). This polar metabolite is formed by deacetylation of the C-4 acetate in fusarenon-X. In vitro studies with liver microsomes also revealed nivalenol as the sole metabolite of fusarenon-X (Ohta et al., 1978).

b. Trichothecene Skeleton

(1) Mice and Rats. In 1979, Nakano et al. reported on the fate of [^{14}C]-labeled 12,13-epoxytrichothec-9-ene in mice and rats. The authors assumed that this trichothecene skeleton (TS), common to all trichothecene derivatives, would have the same basic chemical behavior in vivo as other derivatives but would differ in toxicity. Whole body autoradiography of mice administered TS intravenously at a dose of 9.5 mg/kg showed incorporation of radioactivity in the liver, kidneys, bladder, and urine as early as 10 minutes after dosing. Radioactivity appeared in the contents of the small intestine by 30 minutes and spread aborally for 2 hours after dosing. Radioactivity remained high in the urine but decreased in the liver and kidneys by 2 hours. Similar results were obtained following oral administration of TS at 10.5 mg/kg. Radioactivity appeared in the liver, kidney, bladder, and urine by 0.5 hour, the earliest time point sampled. Thereafter, levels in liver and kidney decreased with time. Radioactivity had appeared in the intestines by 0.5 hours and spread to the cecum by 3 hours and the colon by 12 hours. By 24 hours, very little radioactivity was detected in any tissue.

The distribution of radioactivity was also assessed in mice following the intravenous administration of TS at 9.7 mg/kg b.w. Oxidation of tissues to $^{14}CO_2$ was followed by liquid scintillation counting. As early as 10 minutes after administration, a large amount of radioactivity was present in the liver (13.3% of dose) and gastrointestinal tract (with contents, 13.2% of dose). With the exception of the small intestine and kidneys, which reached maximal levels of radioactivity 1 hour after dosing, all tissue levels of radioactivity declined after 10 minutes. A total of 66.7% of the administered radioactivity was excreted in the urine and 28.0% in the feces by 24 hours. These results were consistent with the previous autoradiography findings and together the studies demonstrated the rapid absorption, distribution, and elimination of TS.

As early as 1 hour after oral dosing of mother mice with TS at 6 mg/kg b.w., radioactivity was detected in the stomac contents of the nursing infant mice indicating transmission through the milk. The amount of radioactivity detected was low, at less than 0.02% of the dose. Some losses were presumed to have occurred from rapid absorption and distribution of the toxin from the gastrointestinal tracts of the infant mice.

The tissue distribution and excretion of radiolabeled TS following intravenous and oral administration were also compared in rats at 6 hours after dosing. A total of 16.2 and 19.3% of the administered radioactivity were excreted in the urine, while at this early time only 0.1 and 0.9% were excreted in the feces after administration by the intravenous and oral routes, respectively. No $^{14}CO_2$ was detected in expired air by either route of administration. The majority of radioactivity was located in the gastrointestinal tract, accounting for 50.0 and 60.6% of the dose given by the intravenous and oral routes, respectively. Major differences between the two routes were reflected in the radioactivity of the brain, spleen, kidneys, testes, and carcass where the levels following intravascular administration were four to five times higher than after oral dosing. No examinations of metabolites were performed in these studies. This is one of the few studies in which different routes of administration using radiolabeled compounds have been directly compared. The higher concentrations in brain, spleen, and kidneys in animals dosed intravenously with toxic trichothecenes may be of toxicological significance.

c. Deoxynivalenol

Studies on the fate of DON have been hindered in part due to the lack of suitable radiolabeled material. No specific synthetic routes for introducing tritium or ^{14}C into DON have been established, and nonspecific routes for introducing tritium have resulted in decomposition of the molecule. As a result, initial whole animal studies investigating the disposition of DON have relied upon gas chromatographic methods of monitoring DON residues. Therefore, studies have focused primarily on the monitoring of the parent compound and in some instances metabolites which display similar chromatographic behavior such as DOM-1. Identification of conjugates by this manner requires liberation of the aglycone with ß-glucuronidase and subsequent gas chromatographic identification of the freed toxin. However, ezymatic incubation also increases the amount of background and as a result raises the limits of detection. These complicating factors have caused many researchers to limit their work to identification of only the parent compound. Recently, biosynthetically labeled [^{14}C] DON has been achieved and several publications on the disposition of DON in several animal species followed.

(1) Rats. The metabolic fate of deoxynivalenol was first studied by Yoshizawa et al. (1983). They detected a novel biotransformation product called DOM-1 as the sole metabolite in urine and feces of rats orally administered DON. DOM-1 had a molecular weight 16 mass units less than DON indicating a loss of oxygen. The 12,13-epoxide was reduced with a corresponding loss of oxygen in

DOM-1 to yield a carbon-carbon double bond. This deepoxy DON metabolite was the first report of the in vivo biotransformation of a trichothecene by reduction of the 12,13-epoxide group.

Only DOM-1 was detected in the urine and feces of rats administered DON orally at a dosage of 2 mg/kg b.w. (Côté et al., 1987). Neither free DON nor conjugates of DON or DOM-1 were found indicating that the more polar trichothecene DON does not appear to undergo conjugation as a pathway of biotransformation in this species.

The metabolism of DON in rats was further investigated by Lake et al. (1987) using [^{14}C]-labeled DON. After a single oral dose of 10 mg/kg DON, 25% and 64% of the administered dose was eliminated within 4 days in the urine and feces, respectively. Very little radioactivity (< 0.1% of the administered dose) was detected in any of the tissues 4 days after toxin administration. The major metabolites observed in the urine were parent DON (25%), DOM-1 (10%), and polar compounds eluting with reverse phase HPLC retention times of less than 4 minutes. The polar metabolites remained uncharacterized, but the authors speculated that they were probably glucuronide conjugates. Metabolic profiles in the feces were qualitatively similar to that of urine. Parent DON accounted for less than 5% of the total residues and DOM-1 only 13%. The major metabolite observed in the feces were the polar compounds (75%) attributed to conjugates.

The discrepancy between the work of Côté et al. (1987), where no conjugates of DON were detected, and the work of Lake et al. (1987), where the predominant residues eliminated by the rats were polar compounds attributed to conjugates, may be due to several factors including: 1) dosage (2 mg/kg vs 10 mg/kg), 2) diet, 3) strain of animal, and 4) the availability of radiolabeled compound. Since the polar DON metabolites reported by Lake et al. (1987) were not specifically characterized, it is also possible that these compounds were not glucuronide conjugates but rather some other novel metabolite. Although the ability of rats to form glucuronide conjugates of DON reported by the various authors has been inconsistant, in all instances DOM-1 was detected in the feces in greater quantities than parent DON.

(2) Poultry. DON-contaminated wheat diets were given to chickens for 28 to 160 days in a feeding study with broiler and laying hens (El-Banna et al., 1983). The concentration of DON in the finished diet was 5 mg/kg. No residues of DON were detected in eggs, drumsticks, breast muscle, liver, or gizzard at a detection limit of 10 ng/g. Decomposition of DON residues during storage was eliminated as a possibility for the nondetection of residues since DON was demonstrated to be stable in tissues for 6 days at 4°C. Similarly, no residues of DON were detected in liver, heart, kidney, breast muscle, or thigh muscle of chickens given diets formulated with deoxynivalenol contaminated wheat diets and containing 0, 9, or 18 mg DON/kg feed for 1 to 35 days (Kubena et al., 1985). Lun et al. (1986) fed White Leghorn hens extremely high levels of DON contaminated feed (82.8 mg/kg) for

27 days. No residues of DON were detected in the yolk, liver, kidney, thigh muscle, or breast muscle even at this high dosage. However, trace levels of DON (approximately 20 ng/g) were detected in the gizzard. The authors postulated that the majority of DON was converted into some unknown metabolite(s) since only 4.2% of the total DON consumed was recovered in the excreta.

In three separate experiments in poultry, no DON residues were detected in eggs of chickens fed diets a variety of DON contaminated feeds. The concentration of DON in the diets and length of time the birds were fed these diets ranged from a low of 18 mg/kg DON from Day 1 of age to onset of egg production (Kubena et al.,1987), 40 mg/kg DON for 58 weeks (Moran et al., 1987) to a high of 82.8 mg/kg DON (Lun et al., 1986). Although no DON residues were detected in eggs or egg fractions (yolk and albumen) in any of the above studies, the presence of potentially toxic metabolites were suggested due to the death of the embryos after egg incubation (Moran et al., 1987).

Two studies have been conducted using [^{14}C]-labeled DON in poultry. Following a single dose of 2.2 mg DON/bird, low concentrations of DON residues equivalent to 1.9 µg/60 g egg were detected (total radioactivity) within 24 hours (Prelusky et al., 1986). Residue levels declined rapidly thereafter. Gas chromatographic/mass spectrometric analysis of the eggs showed that only 10% of the radioactivity present was parent DON.

In a subsequent study, tissue distribution and excretion of radiolabeled DON in hens was examined (Prelusky et al., 1987). The toxin was shown to be poorly absorbed (less than 1% of the administered dose) following a single oral dosage of 2.2 mg DON/bird (2.4 µCi/bird). The average half-life for tissue clearance was 16.8 hours (7.7 to 33.3 depending upon the tissue). The total radioactivity recovered in the excreta was 78.6%, 92.1%, and 98.5% at 24, 48, and 72 hours, respectively.

Accumulation of radioactivity in tissues did not occur in birds dosed with 2.2 mg DON/bird for six consecutive days (Prelusky et al., 1987). Maximum tissue concentrations of total DON residues (total radioactivity) occurred in the kidneys but were only equal to 60 ng/g. GC-MS analysis of the excreta samples revealed only 22 to 52% of the total radioactivity present was parent DON. Much of the remaining radioactivity was polar in nature as it was not extracted with the solvents used and remains uncharacterized.

(3) Swine. In order to assess the pharmacokinetics of DON, Coppock et al. (1985) intravenously dosed two swine with DON at a dosage of 0.1 mg/kg. The plasma disappearance half lives were 2.08 and 3.65 hours for the two swine, respectively. At 24 hours postdosing, no residues of DON were detected in skeletal muscle of either pigs. DON residues were not detected in the liver and were found in the kidney of only one animal at a concentration of 24 ng/g. The 24-hour urinary excretion of the parent compound accounted for 28 and 57% of the DON administered to the two swine. The pharmacokinetics of DON were best described by a

one-compartment open model. In a feeding study with swine, five-week-old piglets were given diets naturally contaminated with DON for 5 weeks (Côté et al., 1985). The feed was amended with corn naturally contaminated with DON to give diets containing 0.7, 3.1, and 5.8 mg DON/kg. Only trace amounts of DON were found in tissues of swine given diets at the higher level of DON (5.8 ppm) and no residues were detected in tissues of animals given diets containing the two lower levels of DON. However, the parent compound, DON, was detected in plasma, urine and gastrointestinal contents of animals fed the high DON diet, with maximal concentrations of 0.10 ppm, 4.32 ppm, and 1.60 ppm, respectively.

In a swine metabolism feeding trial by Friend et al. (1986), seven pairs of littermates were fed one of two experimental diets (control diet and 5.26 ppm DON-contaminated wheat diet) for 5 days. The average daily intake of DON was estimated to be 0.10 mg/kg b.w. A minimum of 67% of the ingested DON was absorbed based upon urinary recovery. Over 90% of the total residues recovered were found in the urine and the predominant compound present was parent DON. Only traces of the deepoxy metabolite DOM-1 were detected.

Recently, Prelusky et al. (1988) examined the pharmacodynamics of [^{14}C] DON in swine following both intravenous and intragastric administration of the toxin. The pharmacokinetics of DON (total radioactivity) displayed a three-compartment open model with half-lives of 5.8, 96.7, and 510 minutes for the rapid distribution, slower distribution, and terminal elimination phases, respectively. The plasma clearance rate was 1.81 ml/min/kg, similar to that found in sheep (Prelusky et al., 1986o). DON was eliminated predominantly in the urine (86 to 104%) and essentially intact (> 95% parent DON). The bioavailability (F) of DON after intragastric administration was calculated to be between 48 and 65%. With both routes of administration, DON was excreted essentially intact. Less than 5% of the total residues recovered were present as glucuronide conjugates and no DOM-1 was detected. The greater systemic absorption of DON by swine, and the lack of any significant metabolism may account for the greater sensitivity of swine to DON compared to other species. For instance, the degree of DON absorption following an oral dose was estimated to be less than 1% in poultry (Prelusky et al., 1985) and dairy cows (Prelusky et al., 1984) and only 6 to 10% sheep (Prelusky et al., 1985,1986).

The failure to detect DON residues in tissues of swine or chickens fed diets naturally contaminated with DON indicates that DON is not significantly transmitted as parent compound into edible tissues. Because of the comparatively low acute toxicity of DON and the apparent lack of transmission into tissue, human consumption of meat from animals ingesting DON through the diet is not likely to pose any significant health threats, although further work should be conducted in order to rule out the possibility of (toxic) metabolite transmission.

(4) Cattle. In 1984, Prelusky et al. reported on the absorption and nontransmission of DON into milk of dairy cattle. Utilizing a gas chromatographic method with electron capture detection, no measurable quantities of DON were detected in the blood, serum or milk of a cow administered a single oral dose of 50 mg pure crystalline DON (0.10 mg DON/kg b.w.). Detection limits for the assay were reported to be 10 ng/ml. A more sensitive gas chromatography-mass spectrometry (GC/MS) method utilizing selected ion monitoring (SIM) was then developed with a detection limit of 1 ng/ml for use in a subsequent study. Two lactating dairy cows were then intubated with Fusarium contaminated corn extracts containing 920 mg DON to provide a dosage of about 1.7 mg DON/kg b.w. In addition to analysis for free DON, analysis for glucuronide conjugates was performed by incubating duplicate samples of blood serum with ß-glucuronidase and comparing free versus total DON residues. The maximal concentrations of total DON (free plus conjugated) at 3.5 and 4.7 hours after dosing were 70 and 200 ng/ml, respectively. By 24 hours, the levels had dropped to less than 2 ng/ml. Glucuronide conjugates of DON represented 24 to 46% of the total residues in serum at 3.5 and 4.7 hours postdosing.

Both conjugated and free DON were also detected in the milk although the levels were very low. The maximum concentration of total DON residues (free plus conjugates) in milk did not exceed 3 ng/ml and residues were only detected in the first two milking periods (8 and 20 hours postdosing).

In a separate study, three dairy cattle were fed a DON containing diet (66 mg/kg) formulated with naturally contaminated corn for 5 days (Côté et al., 1986). All milk, urine, and feces were collected during the time of feeding and for three days following withdrawal of the contaminated feed. Both free DON and its deepoxy metabolite DOM-1 were detected in the urine and feces at all time periods during the 5 days of feeding. Approximately 20% of the DON fed to the animals was accounted for in the feces and urine. DOM-1 was the predominant residue excreted and the overall ratio of DOM-1/DON in excreta was approximately 24:1. Glucuronide conjugates, predominantly DOM-1, were also detected in urine. Detectable concentrations of DOM-1 were recovered in both urine and feces up to 40 hours after the last feeding of DON.

DON was not detected at a detection limit of 1 ng/ml in any milk sample obtained during this feeding trial. DOM-1, however, was found in the milk of all three cows during the 5-day feeding trial. Using a GC-ECD method (Swanson et al., 1986), the maximal concentration detected was 26 ng/ml. After the DON contaminated diet was withdrawn, DOM-1 was detected in only one milk sample 12 hours later, at a concentration of 4 ng/ml. DOM-1 was confirmed by GC-MS analysis and quantitation closely matched the results obtained by GC-ECD (Yoshizawa et al., 1986). The public health significance of DOM-1 and possible conjugates of DOM-1 in milk remains undetermined. Although intestinal microflora may cleave conjugates (if present) to liberate free DOM-1, the toxicity of this deepoxy metabolite to mammals has not been established. Reduction of the epoxide is

presumed to be a detoxification process but further work is needed to confirm this hypothesis.

(5) Sheep. The fate of DON administered to sheep following both oral and intravenous dosing was recently reported by Prelusky et al. (1986). After oral toxin administration at a dosage of 5 mg/kg b.w., 50 to 75% of the dose was recovered in the feces, as both DON and DOM-1. Urinary excretion rates were maximal at 6 to 9 hours after toxin administration, declining exponentially thereafter. Residues detected in urine and bile included DON, DOM-1, and their respective glucuronide conjugates.

Upon administration of DON to sheep intravenously at a dosage of 0.5 mg/kg b.w., the two major metabolites detected in urine were conjugates of DON and DOM-1 (Prelusky et al., 1986). Urinary DON elimination displayed a biphasic pattern with a mean elimination half life of 1.2 hours. Only 66% of the administered toxin was recovered, primarily in the urine. The authors suggested the remainder of the dose was biotransformed into metabolites which are currently unidentified.

The pharmacokinetics of DON after intravenous administration followed a two-compartment open model with a distribution half-life (α) of 12 to 23 minutes and a mean elimination phase half-life of 67 minutes. The plasma clearance rate was calculated to be 1.47 ml/min/kg.

d. Diacetoxyscirpenol

(1) Humans. Diacetoxyscirpenol (DAS, anguidine) is unique among the trichothecenes in that the toxic effects have been well established in humans during phase I and phase II clinical trials for treatment of malignancies. During evaluation of DAS as a chemotherapeutic agent, over 200 people were administered DAS (Goodwin et al., 1978; Bukowski et al., 1982; Thigpen et al., 1981; Yap et al., 1979; Diggs et al., 1978; Murphy et al., 1978). Minimal antitumor activity was displayed and its use was subsequently discontinued. However, in spite of human clinical trials and the natural exposure of livestock via consumption of contaminated feeds, very little is known about the fate of DAS in animals.

(2) Rats. In addition to the two DAS hydrolysis products, MAS and scirpentriol, two new metabolites called DRM-1 and DRM-2 were detected in the excreta of rats administered multiple oral doses of DAS at 2.8 mg/kg b.w. (Sakamoto et al., 1985). As in swine and cattle, DAS was extensively and rapidly metabolized in the rat and the parent compound was detected in neither the urine or feces. Scirpentriol and MAS were detected only in the urine at 4.9% and 3.5% of the administered dose, however, neither metabolite was detected in feces. The two unknowns were quantitatively more significant than the parent compound, MAS, or scirpentriol. DRM-1 and DRM-2 were found in the urine at 9.5 and 7.2% and in the feces at 9.5 and 18.9% of the administered dose, respectively. These two new metabolites were identified by mass and nuclear magnetic resonance spectroscopy as deepoxy MAS and deepoxyscirpentriol (Figure II.3). Although these

compounds are assumed to be detoxification products, supporting toxicological data is not yet available.

(3) Swine. In swine, the kinetic profiles of DAS and its two metabolites monoacetoxyscirpenol (MAS) and scirpentriol were examined after oral administration of 2 mg/kg b.w. (Bauer et al., 1985). All five animals vomited within the first hour. The analysis of blood serum by GC/MS demonstrated the presence of DAS, MAS, and scirpentriol, although at very low concentrations. Maximal serum concentrations of DAS, MAS, and scirpentriol were 21.9 ng/ml, 13.2 ng/ml, and 14.8 ng/ml, respectively. The highest amounts of all three compounds were found 30 to 60 minutes postdosing, and no traces of toxin were detected in any animals after 24 hours of toxin administration.

The pharmacokinetics of parent DAS were evaluated in swine after intravenous administration of 0.1, 0.5, and 1.0 mg/kg b.w. (Coppock et al., 1987). Vomiting occurred in all swine and the time between dosing and onset of vomition decreased with increasing dosages. A large apparent volume of distribution (1.58 +/- 0.62 ml/kg) and a high total body clearance (119.4 ml/min/kg) was demonstrated for DAS in swine dosed 48 hours after anesthesia. Less than 1% of the parent compound was recovered in the urine.

Monoacetoxyscirpenol and scirpentriol were detected as the major biotransformation products; however, kinetic data on these metabolites were not reported. Scirpentriol was the only metabolite detected in selected plasma samples analyzed 8 hours postdosing (Swanson et al., 1984).

The mean DAS plasma disappearance half-life was 10.2 minutes. However, in animals dosed 24 hours after halothane anesthesia, the mean plasma disappearance half-life was four times longer, 39.2 minutes. Upon necropsy, one animal was found to have liver damage (multifocal hepatocellular necrosis, and the nature of the lesion suggested that it was present prior to dosing). This pig displayed a much longer plasma disappearance half-life of 150 minutes. Similarly, the total body clearance was also decreased in animals dosed 24 hours after anesthesia and in the animal with concurrent liver disease.

The finding that hepatic damage increased the plasma half-life was not surprising since the liver is considered the primary site of xenobiotic metabolism in animals. The increase in the plasma disappearance half-life of DAS in swine dosed within 24 hours after halothane anesthesia demonstrated a dramatic effect of exposure to an additional xenobiotic on trichothecene pharmacokinetics. Toxic effects in animals are correlated with plasma toxicant concentrations. Obviously, any disease which decreases the rate of metabolism or excretion of toxins may increase toxicity. Similarly, concurrent treatment with xenobiotics which inhibit biotransformation of trichothecenes to less toxic metabolites (inhibit detoxification) may result in an increase in toxicity. Thus, the results of this study suggest that previous liver damage or agents affecting hepatic

metabolism may not only alter toxicokinetics of trichothecenes but also affect their toxicity.

e. T-2 toxin

T-2 toxin has been studied more than any other trichothecene. One reason for the investigative attention that T-2 toxin has received was the early availability of a stable, specifically labeled radiotracer. Scientific reports on the fate of T-2 in a variety of animal species increased dramatically after Wallace et al. (1977) published a method for producing tritium labeled T-2 toxin. With this technique a nonexchangeable tritium label was introduced at the C-3 position which proved to be superior to biosynthetic incorporation of ^{14}C or ^{3}H into T-2 toxin, in terms of both higher specific activity and reduced cost.

(1) Mice. The metabolic fate of T-2 toxin was first reported in mice and rats by Matsumoto et al. (1978). Both rats and mice were given tritium-labeled T-2 toxin orally (specific activity of 14 mCi/mmol) at 1 mg/kg b.w. Neither the method used to label the T-2 toxin nor the position(s) and stability of tritium incorporation were reported.

The distribution of radioactivity in mice given tritium labeled T-2 toxin was rapid and maximal tissue levels occurred by 30 minutes. The radioactivity of tissues also decreased rapidly and by 72 hours no radioactivity was detected. The highest uptake of radioactivity was observed in the liver followed by the kidneys and spleen. A significant amount of radioactivity was found in the bile and in the gastrointestinal tract. Blood levels showed a biphasic profile with the highest values at 1 and 24 hours after dosing. The majority of radioactivity was located in the serum and not the cells. This biphasic blood profile, in addition to the high levels found in the bile, suggested that enterohepatic recirculation may have occurred. The total radioactivity was excreted by the mice in a feces:urine ratio of 3:1 over a 72-hour period representing a total of 68% of the administered dose.

Utilizing an immunoperoxidase staining technique, Lee et al. (1984) followed the distribution of T-2 toxin at the cellular level in the liver, kidneys and alimentary tract of mice for 24 hours following oral administration of the T-2 toxin at 11 mg/kg b.w. At 5 minutes after dosing, a very high concentration of T-2 toxin was found in the superficial and deep squamous epithelial cells of the esophageal mucosa. T-2 was found in the cytoplasm of gastric mucosal epithelial cells from 25 minutes to 3 hours after exposure and disappeared by 6 hours. In the duodenum, T-2 was detected by 15 minutes in the epithelium of isolated villi. From 25 minutes to 3 hours after dosing the toxin was found in the cytoplasm and nuclei of surface epithelial cells, the cytoplasm of macrophages and neutrophils of the lamina propria, in addition to the nuclei of villous tip fibroblasts. No T-2 was detected in the duodenum 6 hours after exposure. Very little T-2 was detected in the cytoplasm of jejunum villous tip epithelial cells from 40 minutes to 1.5 hours. No T-2 was detected in the ileum even by 24 hours nor in

the liver at any time point. In the kidneys, the majority of T-2 was located in the medulla rather than the cortex. Similar results for the kidneys were obtained by Nakano et al. (19[illegible]) following whole body autoradiography of mice administered [^{14}C]-13-labeled 12,13-epoxytrichothecene (the trichothecene skeleton) by both intravenous and oral routes. The binding affinity of the antisera used in the study described above was reported to be much greater for T-2 than for HT-2, with very little cross reactivity to neosolaniol and T-2 tetraol. The binding affinity of the antisera to hydroxylated metabolites such as 3'-OH T-2 and 3'-OH HT-2 was not assessed. Due to the specificity of the antibodies used in this study, the distribution of peroxidase reaction products were likely to be due primarily to T-2 toxin.

(2) Rats. Rats orally administered tritium labeled T-2 toxin excreted approximately 69% of the administered dose. Compared with mice, rats eliminated a larger percentage of radioactivity in the feces (5:1, feces:urine ratio) and over a shorter period of time (24 hours) (Matsumoto et al., 1978). Silica TLC and column chromatographic analysis revealed the presence of four trichothecenes in the feces; T-2, HT-2, and the two unknowns called U-III and U-IV, accounting for 2.7, 7.5, 25.8, and 9.1% of the dose, respectively. The unknown U-IV remained at the origin of the TLC plates, indicating the metabolite was very polar. Neosolaniol, HT-2, and three unknowns were detected in the urine, each totaling less than 8% of the administered dose. Identification of compounds was based upon TLC Rf values and column chromatographic elution profiles. No parent T-2 was detected in any sample demonstrating the rapidity of T-2 toxin metabolism.

Several novel deepoxy T-2 metabolites in rats were recently reported by Yoshizawa et al. (1985b). Six rats were administered multiple oral doses of either T-2 tetraol or 3'-hydroxy HT-2 at 9 mg/kg b.w. T-2 tetraol was converted to deepoxy T-2 tetraol and excreted in the urine and feces at a ratio of 7:20. The 3'-hydroxy HT-2 was deepoxidated to yield deepoxy-3'-hydroxy HT-2 (DE-3'-OH HT-2), deepoxy-3'-hydroxy T-2 triol (DE-3'-OH T-2 triol) and deepoxy-15-acetyl T-2 tetraol (DE 4-DN) (see Figure II.3). Although known metabolites of T-2 toxin (T-2 tetraol and 3'-hydroxy T-2) were given to the rats in this study rather than T-2 toxin, similar metabolites are likely to be produced. Since T-2 appears to be rapidly biotransformed in all animal species examined to date to give multiple metabolites, it is unlikely that intact deepoxy T-2 would be detected. Instead it would be rapidly biotransformed to give deacylated and/or oxidized metabolites of deepoxy T-2 rather than deepoxy T-2 directly. Deepoxy biotransformation products of several trichothecenes have been detected including T-2 (Corley et al., 1986; Yoshizawa et al., 1985a,b), DAS (Sakamoto et al., 1986), and DON (Yoshizawa et al., 1983, 1985a,b, 1986; King et al., 1985) indicating deepoxidation is an important pathway in metabolism of trichothecenes and deserves further attention.

(3) Guinea pigs. The fate and distribution of tritium labeled T-2 toxin was investigated after IM injection in male guinea pigs

(Pace et al., 1985). Radioactivity was detected in all tissue samples by 30 minutes postdosing indicating rapid absorption of the toxin. The plasma concentration vs. time plots were multiphasic with maximum plasma concentrations occurring by 0.5 hour. By 5 days, 75% of the administered dose was excreted in the urine and feces. The ratio of radioactivity excreted in urine/feces was 4 to 1. The majority of radioactivity excreted into the urine occurred during the first 24 hours and declined rapidly thereafter. In contrast, radioactivity slowly increased in the feces during the first 5 days. By 28 days, only trace amounts of radioactivity (0.04% of dose) were detected in the excreta.

No parent T-2 toxin was detected in any urine, blood, or tissue sample. In plasma, HT-2, 4-deacetylneosolaniol, T-2 tetraol, and several polar conjugates were detected using thin-layer radiochromatography. In urine, T-2 tetraol, 4-deacetylneosolaniol, 3'-hydroxy HT-2 were identified in addition to several polar unknown metabolites which remained at the origin of the TLC plates. Of all samples analyzed, bile contained the greatest concentration of total trichothecene residues. The major metabolite detected in bile was 4-deacetylneosolaniol along with smaller amounts of HT-2, 3'-hydroxy HT-2, 3'-hydroxy T-2 triol, and T-2 tetraol. As in the urine, a substantial amount of unidentified polar metabolites remaining at the origin of the TLC plates (presumably glucuronide conjugates) were also detected.

(4) Chickens. The distribution of tritium-labeled T-2 toxin (labeled using the procedure of Wallace et al., 1977) in broiler chicks was first described by Chi et al. (1978). The birds were fed diets containing 0, 0.5, 2, or 8 ppm nonradioactive T-2 toxin from 1 to 6 weeks of age and then intubated into the crop with tritium-labeled T-2 toxin at dosages of 0.126, 0.500, and 1.89 mg/kg b.w. The average recoveries of administered radioactivity ranged from 95 to 112%.

Up to 8 hours after dosing, the majority of radioactivity was found in the gastrointestinal tract, including contents. Of the radioactivity in the gastrointestinal tract, the majority was found in the crop and gizzard at 0.5 hour after dosing; in the crop, gizzard, and small intestine at 4 hours after dosing; and thereafter in the large intestine and ceca. From 12 to 48 hours, the majority of radioactivity was found in the excreta. These data indicated very rapid movement of T-2 and its metabolites through the gut and into the excreta. Total radioactivity in blood, plasma, abdominal fat, carcass, heart, kidney, and liver peaked at 4 hours after dosing while the radiolabel in the muscle, skin, bile, and gallbladder reached their maximum by 12 hours. The pattern of excretion and relative tissue distributions over 24 hours were very similar across the three dosage groups.

T-2 toxin and/or metabolites were eliminated primarily through the bile and into the gastrointestinal tract. Since chickens eliminate urine and feces together, however, the relative radioactivity in the excreta actually contributed by the kidney

or digestive tract could not be separately determined. The authors postulated that humans would be unlikely to be affected by consuming the muscle from chickens fed diets containing concentrations of T-2 toxin likely to occur in natural outbreaks. A similar conclusion was reached by Hoffman (1980). No carry over of T-2 toxin or HT-2 could be demonstrated in the edible tissues of roosters given a diet amended with 15 ppm T-2 toxin when analyzed using a two-dimensional TLC method with a detection limit of 15 ng/g. In the liver, the maximum concentrations of T-2 was reached between 2.5 and 3 hours and disappeared by 5 hours. The metabolite HT-2 was detected for only a few hours longer.

In 1980, Yoshizawa et al. described the detection of metabolites in the excreta of broiler chickens given tritium labeled T-2 toxin. They extracted the excreta with acetonitrile followed by partitioning against petroleum ether to remove nonpolar interferences. The acetonitrile layer was then concentrated, and the toxin residues were purified on Amberlite XAD-2 columns and florisil columns. Final separation of residues was accomplished on C18 cartridges. The metabolites were fractionated by successive elution with water and 20, 50, 70, and 100% methanol. Aliquots of individual C18 eluates were analyzed by high performance silica gel thin-layer radiochromatography. Selected extracts were analyzed by gas-liquid chromatography (packed column) using a hydrogen flame detector and by GC-MS following derivatization to trimethylsilyl (TMS) ethers or trifluoracetyl (TFA) esters. The mean recoveries of radiolabel from control excreta spiked with T-2 toxin following TLC analysis was 73.6% when added at 0.2 and 3.1 µg [^{3}H] T-2/g of excreta.

In this study, [^{3}H]-labeled T-2 toxin (synthesized by the method of Wallace et al., 1977, with a specific activity of 100.6 µCi/mg) was administered as a single oral dose of 1.6 mg/kg (3.53×10^8 dpm/kg) to 47-day-old broiler chickens which had been fed a diet containing 10 µg of nonradioactive T-2 toxin per g of ration for 5 days. A total of 19.31, 29.95, and 80.18% of the administered dose was excreted by the chickens at 4, 12, and 48 hours after dosing, respectively. Several metabolites, in audition to the parent T-2 toxin were isolated including: neosolaniol, HT-2, T-2 triol, and T-2 tetraol. All but triol were confirmed by GC-MS. Several unknown metabolites called TB-1 through TB-8 were found to be quantitatively much more significant. TB-6 was determined by GC/MS analysis to be 4-deacetylneosolaniol (4-DN). The sum of these metabolites represented 10.16, 13.47, and 55.20% of the administered dose and 52.62, 44.97, and 68.85% of the total radioactivity excreted by the chickens at 4, 12, and 48 hours after dosing, respectively.

Significant losses of radioactivity had occurred at the initial acetonitrile extraction of the excreta 48 hours after dosing. Approximately 10% of the administered radioactivity remained in the residue. The XAD-water eluate accounted for as much as 15% and the florisil-methanol eluate represented as much as 24% of the extracted radioactivity. The majority of radioactivity from

the XAD-water and florisil-methanol eluates remained at the origin of the plates following TLC analysis, indicating the compounds were much more polar in nature than the parent T-2 toxin.

Several of the unknown T-2 metabolites detected in the above mentioned chicken radiotracer experiments were subsequently identified by Visconti and Mirocha (1985a). TB-1 and TB-2 were determined to be the same compound, 3'-hydroxy T-2 toxin. TB-3, the major metabolite present, corresponded to 3'-hydroxy HT-2 toxin. TB-4 and TB-5 corresponded to 8-acetoxy and 15-acetoxy T-2 tetraol (also called 4-deacetylneosolaniol) in addition to another monoacetylated isomer of T-2 tetraol whose structure is currently unidentified. TB-7 and TB-8 remain unidentified as of this writing but may have represented glucuronide conjugates.

In addition to excreta, several organs were also analyzed to determine which metabolites were present. The radioactivity detected in chicken livers by Chi et al. (1978) was proposed by Visconti and Mirocha (1985a) to be comprised of 3'-hydroxy HT-2 (the major compound), HT-2, and T-2 triol in addition to small amounts of T-2, 15-acetoxy T-2 tetraol, 4-acetoxy T-2 tetraol and T-2 tetraol. Unfortunately, no recoveries were given to permit the reader to assess the amount of metabolite residues accounted for by the compounds. Traces of HT-2 and 3'OH HT-2 were found in the lungs, but no trichothecenes were detected in the heart and kidneys which is in agreement with a previously reported radiotracer experiment (Chi et al., 1978).

(5) Cattle. The pharmacokinetics of T-2 toxin were examined in female calves by Beasley et al. (1986). Following intravenous administration of T-2 toxin the plasma disappearance half-life was 17.4 minutes and the mean apparent specific volume of distribution was 0.376 L/kg. The elimination of T-2 followed a two-compartment open model. Negligible quantities of the T-2 were eliminated as the parent compound, less than 0.1% of the dose was recovered in urine as T-2.

When calves were dosed orally with crystalline T-2 toxin at 2.4 and 3.6 mg/kg b.w., no parent compound was detected in plasma, urine, or feces at a detection limit of 25 ppb, in spite of the development of severe clinical signs of toxicosis (somnolence, prolonged recumbency, abdominal straining, forced exhalation, and slow capillary refill). At approximately 24 hours postdosing, however, trace amounts of T-2 were detected in ruminal contents, but no parent compound was detected in the lower gastrointestinal tract. No T-2 toxin was detected in any tissues from calves dosed either intravenously or orally including one calf given a lethal intravenous dose which died at 10.5 hours postdosing and another animal given a single oral dose of 0.6 mg/kg and killed at 6 hours postdosing. The lack of parent T-2 residues in animal tissues and excreta shortly after administration of a lethal dose of toxin demonstrates the extent and rapidity that T-2 is biotransformed in animals, and particularly cattle.

The metabolic fate of T-2 toxin was evaluated in a pregnant, lactating cow by Yoshizawa et al. (1981). In this study, tritium-labeled T-2 (specific activity of 190.1 mCi/mmol) was administered orally in a gelatin capsule as a powder at a dose of 0.418 mg/kg (64 mCi, total) to a single 375 kg lactating Jersey cow. The cow had been dosed orally with 100 mg T-2 toxin/day for the previous 3 days. The procedure used to extract the excreta and quantitate metabolites was the same as that reported by Yoshizawa et al. (1980). Extraction of metabolites from plasma was accomplished using Amberlite XAD-2 resin columns. Metabolites in milk were extracted with acetone, partitioned against petroleum ether, and successively chromatographed with Amberlite XAD-2 and florisil columns as with excreta.

At 72 hours, the cow was killed, and approximately 72% of the administered radioactivity was eliminated in the feces and 29% in the urine. Only 0.2% of the radioactivity was detected in the milk. Peak concentrations of total toxin resisues (total radioactivity) for urine (5.5 ppm) and milk (37 ppb) were reached by 16 hours, for plasma (64 ppb) by 8 hours, and feces (9.2 ppm) by 44 hours. Elimination phase half-lives for total tritium residues in urine, plasma, and milk were 12, 16, and 24 hours, respectively. For comparison, it may be noted that the elimination phase half-life for only the parent compound, T-2 toxin, from plasma of calves administered T-2 intravenously was reported to be 17.4 minutes by Beasley et al. (1986). Since the elimination of radioactivity was nearly complete by 72 hours in the study of Yoshizawa et al. (1981), the levels detected in excreta, tissues, and body fluids obtained at necropsy were quite low. These levels (ppb of T-2 equivalents represented by total radioactivity) at 72 hours were as follows: urine--212.0, feces--136.9, bile--27.2, liver--18.5, kidney--13.9, whole blood--13.3, plasma--10.2, mammary gland--11.3, milk--11.4, ovaries--10.7, heart--10.1, spleen--9.4, muscle--8.8, and fat--4.7 ppb.

Due to the extremely low total radioactivity in tissues, metabolites were identified only in urine, feces, milk, and plasma specimens. In addition to a small amount of unmetabolized T-2 toxin, several metabolites were identified in urine including HT-2 toxin, neosolaniol, and 4-deacetylneosolaniol (4-DN). Several unknown metabolites labeled TC-1, TC-3, and TC-5 through TC-8 were also identified with TC-1, TC-3, and TC-6, representing approximately 40% of the total metabolite residues in urine at 12 hours after dosing. TC-6 and TC-3 were also detected in the stomach contents, liver, and amniotic fluid of the cow fetus (Mirocha, 1983). T-2 metabolites are therefore able to cross the placenta into the fetal circulation in the cow.

In feces, no parent T-2 or TC-1 were found. The major free metabolites at all time periods were TC-3, TC-6, and 4-DN. The major metabolites detected in plasma were TC-1, TC-3, TC-6, and TC-8, which together represented a total of 51% of the extractable tritium residues at all time points in the study. Plasma concentrations of unmetabolized T-2 toxin in addition to

HT-2, 4-DN, and neosolaniol were less than 1 ppb within 20 hours after dosing.

The major metabolites in milk were TC-1, TC-3, and TC-8, accounting for a total of 60 to 70% of extractable tritium residues up to 36 hours after dosing. Very little unmetabolized T-2 was present (less than 0.1 ppb) by 36 hours after dosing.

In a separate study on the transmission of T-2 toxin into bovine and porcine milk (Robison et al., 1979), unmetabolized T-2 was found in the milk of a pregnant Holstein cow (third trimester) at levels ranging from 1 to 160 ppb during a 15-day period during which the cow was orally dosed at 182 mg of T-2/day. No data was presented on the time course for the elimination of T-2 into the milk after termination of toxin administration and only the parent compound was monitored. Transmission of T-2 toxin into milk, however, appears to be minimal and short exposures of dairy cattle to T-2 contaminated feed is not likely to pose any significant health risk to humans consuming the milk.

In the study of Yoshizawa et al. (1981), up to 54% of the total tritium residue in urine, 19% in feces, 28% in plasma, and over 20% in milk were eluted in the XAD-water fraction and remained unidentified. The relative amounts of radioactivity in this fraction generally increased with time after dosing. These polar metabolites have not yet been identified but were most likely conjugates of T-2 and its metabolites.

The structures of several of the unknown metabolites isolated in the above study have subsequently been characterized. The major metabolites, TC-1 and TC-3, were identified as 3'-hydroxy T-2 and 3'-hydroxy HT-2 toxins, (Yoshizawa et al., 1982), the same metabolites identified in chickens given T-2. TC-6 was identified as the double oxidation product 3'-hydroxy-7-hydroxy-HT-2 toxin (Pawlosky and Mirocha, 1984).

More recently, a new metabolite of T-2, labelled ISO TC-1, was identified as a urinary metabolite in cattle orally administered T-2 toxin. This metabolite was identified by mass spectroscopy as [3,15-diacetoxy-4α-hydroxy-8α-(3-methyl-3'-hydroxybutyryloxy)-12,13-epoxytrichothec-9-ene], an isomer of 3'OH T-2 labelled here as 3-Ac-3'OH HT-2 (Visconti et al., 1985b). Whether the C-3 acetyl group was derived from rearrangement of the C-4 acetyl group found in 3'OH T-2 or via acylation of the C3 position in 3'OH HT-2 remains to be established.

Chatterjee et al. (1986a,b) dosed a single cow orally with 1.88 mg/kg T-2 toxin. After 48 hours the animal was dosed a second time. 3-Acetyl-3'-hydroxy HT-2 (Iso TC-1) was one of the major products in urine collected from the cow. Other major urinary elimination products were deepoxy T-2 tetraol and deepoxy 3'-hydroxy HT-2 (CHatterjee et al., 1986a,b). No T-2 toxin was detected in any of the urine samples, which was consistant with the findings of Beasley et al. (1986). Of particular interest was the observation that the concentration of 3-acetyl-3'-hydroxy HT-2 increased dramatically after the second dose of T-2 toxin, whereas 3'-hydroxy HT-2 was the predominant metabolite after the

first dose. This data adds additional evidence to the hypothesis that dose and, more importantly, frequency of dose are important factors in the biotransformation and elimination of trichothecene mycotoxins.

The C-3' oxidation products of T-2 toxin (3'-hydroxy T-2, 3'-hydroxy HT-2, 3'-hydroxy triol) have been identified as major elimination products of T-2 in several animal species. Although traces of 3'-hydroxy-7-hydroxy HT-2 were detected in bile of rats perfused with T-2 (Gareiss et al., 1986) and 3-acetyl-3'-hydroxy HT-2 was found as a very minor component in the exceta of chickens orally administered T-2, to date these two oxidation products have been detected as major metabolites only in bovine specimens.

(6) Swine. In 1979, Robison et al. reported on the distribution of tritium-labeled T-2 toxin in swine. The labeled T-2 toxin (prepared by the method of Wallace et al., 1977) was administered orally to two weanling crossbred pigs at doses of 0.1 mg/kg b.w. (2.41×10^9 dpm) and 0.4 mg/kg b.w. (1.22×10^{10} dpm). The distribution of radioactivity in the tissues at 18 hours was very similar to that reported in chickens by Chi et al. (1978) with the exception that the kidneys of swine had a slightly higher level of radioactivity per g of tissue than the liver, which was just the opposite of chickens. The total radioactivity in the liver of swine was higher than the total in the kidneys. Less than 50% of total radioactivity was recovered in this study; the remainder was thought to be in the gastrointestinal tract and its contents. No identification of metabolites was attempted. Unmetabolized T-2 toxin was found to be transmitted into the milk of a sow fed a diet containing 12 ppm T-2 toxin for 220 days (Robison et al., 1979b). Although only one milk sample was analyzed, this sample was taken 6 days after parturition (day 190 of the feeding study) and was found to contain 76 ppb T-2 toxin. No attempt was made to identify metabolites. The mean plasma elimination half-life following intra-aortal administration of T-2 toxin (0.3, 0.6, and 1.2 mg/kg) to swine was 13.8 minutes and followed a two-compartment open model (Beasley et al., 1986). The mean apparent specific volume of distribution was similar to calves, 0.366 L/kg. Negligible quantities of T-2 were eliminated in the urine or feces as the parent compound. Analysis of tissues of swine killed from 1 to 4 hours after dosing at 1.2 mg/kg b.w. revealed rapid disappearance of the parent toxin. The lymphoid organs, in particular the spleen and mesenteric lymph nodes contained the highest concentrations of parent compound with a maximum value of 160 ng/g. T-2 toxin was detected in bile from only one animal (less than 40 ppb) and no parent compound was found in any specimens of liver or adipose tissues.

Neither T-2 toxin nor metabolites were detected in the blood or urine of swine topically administered T-2 toxin at 15 mg/kg b.w. (Pang et al., 1987) when samples were collected at 1, 3, 7, and 14 days postdosing. In addition, no free T-2 toxin or metabolite residues were found in bile. Selected bile and urine samples were also analyzed after treatment with ß-glucuronidase to free conjugates followed by alkaline hydrolysis of the

extracts to give the parent alcohol, T-2 tetraol (Rood et al., 1986,1988). Quantitation was accomplished by gas chromatography with electron capture detection (GC/ECD). Two urine and one bile sample yielded T-2 tetraol after the enzymatic chemical hydrolysis, indicating that swine have the capacity to form glucuronide conjugates or T-2 toxin (or metabolites) after dermal administration. The identification of individual conjugated metabolites was not reported.

Far more prominent were the amounts of parent toxin and/or deacetylated metabolites in the skin of these pigs. In contrast to blood and urine, T-2 toxin was detected in all swine skin and fat samples taken at the site of dermal application. The mean concentrations of T-2 for days 1, 3, 7, and 14 postdosing were 220, 247, 220, and 42 ppm in the skin and 34, 28, 32, and 3 ppm in the fat, respectively. In addition to the parent compound, HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol, and T-2 tetraol were also present in skin and the polar metabolites such as T-2 tetraol increased in concentration over time. The identification of simple hydrolysis products of T-2 toxin in skin demonstrated the capability of local esterase enzymes to biotransform T-2. The rate of absorption and transformation were apparently low since significant amounts of T-2 remained unmetabolized at 14 days after dosing. This was consistent with observations that T-2 was metabolized to HT-2 by human and guinea pig skin in vitro, although in that study the majority of toxin remained intact (Kemppainen et al., 1984).

From a diagnostic point of view the above findings indicate that skin may serve as a potential source for confirmation of topical exposure to trichothecenes particularly when analyzed for the parent toxin. Toxin concentrations within a visible lesion should be much greater than those present in blood or urine and therefore chemical confirmation of dermal exposure would be much easier. The skin and underlying fat apparently act as a depot for the toxin, with absorption occurring slowly over many days. The C3'-hydroxylated T-2 metabolites, 3'OH T-2, and 3'OH HT-2, were not detected in any skin sample from the topically dosed swine indicating mixed function oxidase activity is not involved to any great extent in the biotransformation of T-2 toxin in swine skin, at least at the high concentrations of T-2 toxin used in the study described above.

The disposition of radiolabeled T-2 toxin in swine was investigated after intravascular administration (Corley et al., 1985). Two swine were administered tritium labeled T-2 toxin at 0.15 mg/kg b.w. and the distribution of radioactivity was monitored for 4 hours. The plasma elimination half-life for total tritium residues was 90 minutes. The majority of radioactivity was detected in the urine and the gastrointestinal tracts. Other tissues accounted for only 5% of the administered dose 4 hours after toxin administration. Thin-layer radiochromatographic analysis demonstrated that the parent compound was present only at negligible concentrations, never exceeding 0.25% of the total metabolite residues present in bile or urine. The rapid disappearance of T-2 from animals was therefore not the result of urinary excretion of the parent compound but rather

rapid and extensive biotransformation. Free (unconjugated) metabolites represented less than 20% and 31% of the total metabolite residues in bile and urine respectively. The major free metabolites in both specimens were 3'OH HT-2 and T-2 triol. Glucuronide conjugates of T-2 and metabolites represented 77% and 63% of the total toxin residues present in bile and urine. The major conjugated metabolites were glucuronides of T-2, HT-2, 3'OH T-2, and 3'OH HT-2, respectively. Neosolaniol, 4-DN, and T-2 tetraol in addition to several unknowns were identified, both free and as conjugates. Conjugation of T-2 and metabolites occurred very rapidly. Even in blood samples taken 10 minutes after dosing, 50% of the 3'OH T-2 and HT-2 present in the samples had already been conjugated.

Following reverse phase HPLC separation, the parent T-2 toxin and 20 of its metabolites were detected in the tissues and gastrointestinal tracts of the swine. The predominant metabolites detected included: HT-2, deepoxy HT-2, T-2 triol, deepoxy T-2 triol, 3'OH HT-2, 3'OH T-2, and T-2 tetraol (Corley et al., 1986). The major metabolite in tissues, labeled PM-XV, was not identified, although hydrolysis of this compound yielded deepoxy tetraol, demonstrating that the compound no longer retained the 12,13-epoxide group. Other major metabolites in bile and urine including 3'OH T-2, HT-2, and 3'OH HT-2 were also major metabolites in plasma, tissues, and gastrointestinal tract contents. One of the free metabolites in bile and urine initially identified as T-2 triol by thin layer radiochromatography (Corley et al., 1985) was subsequently found to be comprised of two compounds upon reverse phase HPLC analysis (T-2 triol and an unknown). This new metabolite, identical to the major residue detected in tissues and called PM-XV, was tentatively identified as deepoxy 3'-OH HT-2. This compound was also recently identified as a metabolite in the excreta of rats administered 3'-OH HT-2 (Yoshizawa et al, 1985b).

As demonstrated above, deepoxy trichothecenes are poorly separated from their corresponding epoxidated trichothecenes by normal phase TLC. These congeners are, however, separated by reverse phase HPLC, by reverse phase TLC and by gas chromatography (Yoshizawa et al., 1985a,b; Sakamoto et al., 1985). It is clear that analyses of animal tissues or fluid extracts by TLC can lead to incorrect conclusions due to incomplete separation of deepoxy metabolites from their parent epoxidated analogs. The use of reverse phase HPLC separation is therefore highly recommended for use in future studies pertaining to the fate (distribution, metabolism, and excretion) of trichothecenes in animals.

(7) Humans. There is currently little or no data available pertaining to the absorption, distribution, and metabolism of trichothecenes in humans. Limited information on the in vitro metabolism of T-2 toxin by human cell lines or tissue homogenates was presented in an earlier section. Although over 200 patients were administered DAS in phase I and phase II clinical trials for treatment of human malignancies, no information on pharmacokinetics or metabolism was reported.

Minimal antitumor activity was reported in these studies, and its use was discontinued.

Recently, T-2 toxin along with DON, nivalenol, and DAS have been implicated as components of an alleged chemical warfare agent in Southeast Asia called "Yellow Rain" (Watson et al., 1984; Mirocha et al., 1983). T-2 toxin and/or its metabolite HT-2 were detected (in low concentrations) in 18 blood and three urine samples from alleged victims. In addition, T-2 toxin and HT-2 were found in the heart, stomach, kidney, lung, and intestines of an individual who reportedly died as a result of exposure one month earlier to the "Yellow Rain" chemical warfare agent. DAS was also detected in the kidney of this victim. The finding of relatively high levels of trichothecenes in tissues 1 month after putative exposure has received controversial review and is not consistent with the relatively rapid excretion of trichothecenes displayed in a variety of animal studies.

In addition to trichothecenes, high concentrations of aflatoxin B1 were also detected (12 to 23 ng/g) in the tissues (Watson et al., 194). The natural occurrence of aflatoxin in foods consumed in Southeast Asia has been well documented. Natural occurrence of trichothecenes in Southeast Asia has not been investigated and cannot be ruled out as a source of exposure.

How aflatoxin may affect the metabolism, distribution, and excretion of trichothecenes, regardless of the source of exposure, is not known. Since the liver is a primary organ involved in the metabolism and excretion of trichothecenes, any toxicant (such as aflatoxin) which adversely affects liver function may delay excretion and inhibit metabolism resulting in greater than normal blood and tissue concentrations. An example of this was mentioned earlier herein, pertaining to a study on DAS pharmacokinetics in swine. One animal (dosed with DAS) which was found to have apparently preexistent liver damage upon examination after necropsy, displayed a plasma disappearance half-life approximately 10 times longer than animals with normal liver function (Coppock et al., 1987). Due to the political nature surrounding trichothecene exposure in Southeast Asia and the alleged use of trichothecenes as chemical warfare agents, detection of trichothecene residues in human tissues, urine, or blood is certain to remain a controversial topic.

5. Conclusion

Accurate pharmacokinetic data after the oral administration of trichothecenes is difficult to obtain with many animal species due to the emetic action of these toxins. Vomition after dosing results in inconsistent losses of toxin which can be difficult to account for and which therefore lead to errors in kinetic modeling. As a result, intravenous administrationly is preferred for pharmacokinetic studies because of the resultant total bioavailability of the compound administered. Intravenous administration of trichothecenes, however, also has disadvantages. Intravenous administration of sufficiently high doses of trichothecene toxins results in oliguria or anuria which may be more severe than that which occurs after equivalent oral doses. The severity of renal shutdown appears to be dose-related. Oliguria makes

analysis of urine difficult or impossible due to limited sample sizes. In addition, the effects of renal shutdown on the excretion and kinetics of trichothecenes (and their metabolites) are unknown.

Information to date indicate orally or parentally administered trichothecenes do not accumulate in the body of animals to any significant extent and residues are rapidly excreted within a few days after exposure. Human consumption of edible tissues or milk from animals consuming low levels of trichothecenes is, therefore, unlikely to pose significant health risks, especially if the contaminated feed is withdrawn for a short time prior to milking or slaughter.

Hydrolysis of esters appears to be a major pathway in the metabolism of trichothecenes containing esterified side chains such as DAS or T-2, with hydrolysis of the C4 ester the primary site of attack. Initial hydrolysis cannot be considered significant detoxification since the C-4 hydrolysis products MAS and HT-2 have similar toxicity to their parent compounds DAS and T-2, respectively. Further hydrolysis by esterases to yield the parent alcohols, scirpenetriol and T-2 tetraol, reduces but still does not eliminate toxicity. Exposure to environmental chemicals such as organophosphate pesticides which inhibit esterase activity would likely result in increased trichothecene toxicity by inhibiting this type of detoxification reaction.

Oxidation of trichothecenes has been reported only for T-2 toxin (hydroxylation of C-3' position in many species or C-7 position in cattle). The cytochrome P450 catalyzed oxidation of the isovaleryl group in T-2 appears to be ubiquitous among mammals, at least in a qualitative manner. Hydroxylation of T-2 toxin has been reported to occur in many species including rats, mice, swine, rabbits, guinea pigs, chickens, and cows. As with hydrolysis reactions of trichothecenes, the initial oxidation at the C-3' carbon of T-2 toxin does not significantly reduce toxicity but may accelerate further detoxification reactions.

Recently, reduction of the 12,13-epoxide group has also been reported for several trichothecenes including T-2, DAS, and DON. Although toxicological data pertaining to the deepoxidated trichothecenes is limited, the epoxide is considered essential for toxicity, therefore reduction of the epoxide is assumed to be a form of detoxification. Reduction of the epoxide is probably the product of microbial biotransformation by microflora present in the rumen or gastrointestinal tract. The extent of this reaction among species is still not understood. Nevertheless, recent evidence suggests deepoxidation is a prominent reaction and may be a much more metabolically significant reaction than previously anticipated. The trichothecene biotransformation pathway of epoxide reduction has only recently been described. The importance of this reaction in the overall metabolism and ultimate toxicity of trichothecenes remains to be established. Deepoxy trichothecenes have been characterized only recently and analytical standards of these compounds are not yet readily available. In addition, deepoxy compounds separate poorly from their parent epoxides by TLC. It is, therefore, impossible to speculate on the extent which deepoxydation reactions may have occurred in previous studies on the fate of trichothecenes.

Glucuronide conjugation is another prominent pathway for metabolism of trichothecenes and has been reported for DAS, T-2, and DON and/or their

corresponding metabolites. Conjugation with glucuronides occurs with many different types of compounds and many drugs are conjugated to a significant extent. Glucuronide conjugates are more water soluble and, due to the ionizable sugar, are less likely to diffuse across membranes restricting distribution. The end result is biological inactivation and increased rates of excretion for conjugated trichothecenes. Following passage into the gastrointestinal tract via the bile, conjugates may be cleaved by intestinal microflora liberating the trichothecene aglycone and restoring toxicity of the deconjugated toxin (or metabolite). Reabsorption can then occur to complete the process of enterohepatic recirculation, which may potentially cause delayed excretion and ultimately increased toxicity. Gut microflora may, therefore, play a multiple role in metabolism of trichothecenes, reductive deepoxidation of the 12,13-epoxide and hydrolysis of glucuronide conjugates. In toxicological terms, these reactions (most likely) oppose each other; deepoxidation reduces toxicity, whereas glucuronide hydrolysis restores toxicity.

Although all four major pathways of metabolism (oxidation, reduction, conjugation, hydrolysis) have been identified with the class of trichothecene mycotoxins, T-2 toxin is the only trichothecene for which it has been shown that all four pathways occur simultaneously in the same animal. It is, therefore, not surprising that the metabolism of T-2 toxin is very complex. To date, 26 metabolites of T-2 toxin have been identified by a variety of researchers, including work presented in the following sections of this thesis.

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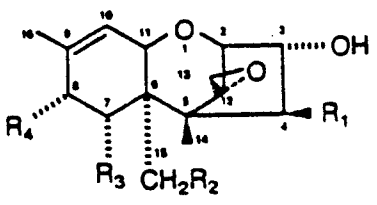
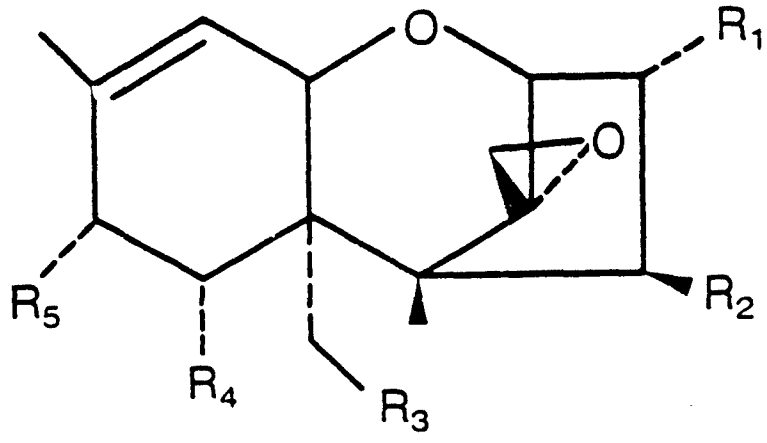
Figure II.1 Structure and numbering system of naturally occurring trichothecenes.



Compound	R1	R2	R3	R4
T-2	OAc	OAc	H	ISV
DAS	OAc	OAc	H	H
NIV	OH	OH	OH	O
DON	H	OH	OH	O
FUS	OAc	OH	OH	O

ISV = $OCOCH_2CH(CH_3)_2$

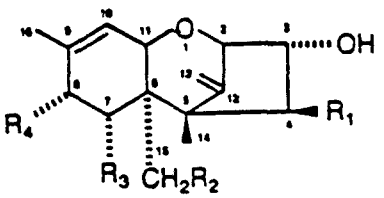
Figure II.2 Structures of T-2 toxin, DAS, and their metabolites.



Compound	R1	R2	R3	R4	R5
T-2	OH	OAc	OAc	H	ISV
HT-2	OH	OH	OAc	H	ISV
4-DN	OH	OH	OAc	H	OH
NEO	OH	OAc	OAc	H	OH
TOL	OH	OH	OH	H	OH
4-Ac TOL(15-DN)	OH	OAc	OH	H	OH
8-AC TOL	OH	OH	OH	H	OAc
3'OH T-2	OH	OAC	OAc	H	ISV-OH
3'OH HT-2	OH	OH	OAc	H	ISV-OH
3'OH TRIOL	OH	OH	OH	H	ISV-OH
3'OH-7-OH HT-2	OH	OH	OAc	OH	ISV-OH
3-Ac-3'OH HT-2	OAc	OH	OAc	H	ISV-OH
3-Acetyl T-2	OAc	OAc	OAc	H	ISV
3-Acetyl HT-2	OAc	OH	OAc	H	ISV
DAS	OH	OAc	OAc	H	H
15-MAS	OH	OH	OAc	H	H
4-MAS	OH	OAc	OH	H	H
STRIOL	OH	OH	OH	H	H

ISV = $OCOCH_2CH(CH_3)_2$; ISV-OH = $OCOCH_2COH(CH_3)_2$

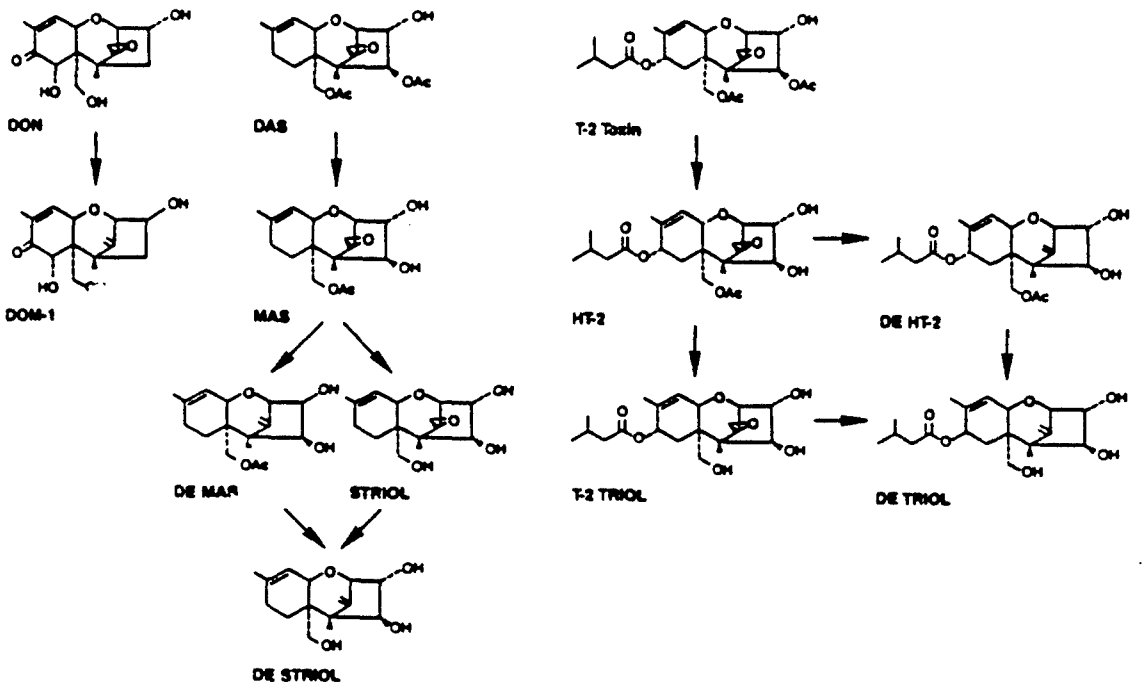
Figure II.3 Deepoxy metabolites of trichothecenes.



Compound	R1	R2	R3	R4
DOM-1	H	OH	OH	=O
DE MAS	OH	OAc	H	H
DE STRIOL	OH	OH	OH	H
DE HT-2	OH	OAc	H	ISV
DE TRIOL	OH	OH	H	ISV
DE TOL	OH	OH	H	OH
DE 4-DN	OH	OAc	H	OH
DE 3'OH HT-2	OH	OAc	H	ISV-OH
DE 3'OH TRIOL	OH	OH		ISV-OH

ISV = $OCOCH_2CH(CH_3)_2$; ISV-OH = $OCOCH_2COH(CH_3)_2$

Figure II.4 Proposed pathway for the metabolism of trichothecenes by bovine lumen microflora.



B. Metabolism

1. In vitro

a. The role of intestinal microflora in the metabolism of trichothecene mycotoxins

by

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Abstract

The role of fecal and intestinal microflora on the metabolism of trichothecene mycotoxins was examined in this study. Suspensions of microflora obtained from the feces of horses, cattle, dogs, rats, swine, and chickens were incubated anaerobically with the trichothecene mycotoxin, diacetoxyscirpenol (DAS). Microorganisms from rats, cattle, and swine completely biotransformed DAS, primarily to the deacylated deepoxidation products, deepoxy monoacetoxyscirpenol (DE MAS) and deepoxy scirpentriol (DE SCP). By contrast, fecal microflora from chickens, horses, and dogs failed to reduce the epoxide group in monoacetoxyscirpenol (MAS) and scirpentriol (SCP), in addition to unmetabolized parent compound. Intestinal microflora obtained from rats completely biotransformed DAS to DE MAS, DE SCP, and SCP; and T-2 toxin to the deepoxy products, deepoxy HT-2 (DE HT-2) and deepoxy T-2 triol (DE TRIOL). Rat intestinal microflora also biotransformed the polar trichothecenes, T-2 tetraol and scirpentriol, to their corresponding deepoxy analogs. Deepoxy T-2 toxin (DE T-2) was synthesized from T-2 toxin and demonstrated to be 400 times less toxic than T-2 toxin in the rat skin irritation bioassay and nontoxic to mice given 60 mg/kg intraperitoneally. Since deepoxidation is a significant detoxification reaction for trichothecenes, variation in intestinal microflora among animals may account, at least in part, for the species variability in toxicity.

Introduction

Trichothecene mycotoxins are a group of sesquiterpenoid compounds produced by a variety of fungi, particularly species of the genus Fusarium. Three of the more agriculturally important members of this toxin group are T-2 toxin, diacetoxyscirpenol (DAS), and deoxynivalenol (DON). All three compounds have been detected in naturally contaminated feeds and food products (Côté et al., 1984; Mirocha, et al., 1977). Consumption of trichothecene contaminated feeds by animals can result in adverse health effects including: feed refusal, diarrhea, emesis, decreased immune response, and death (Côté et al., 1984; Obara et al., 1984; Mirocha et al., 1977; Rosenstein et al., 1979).

T-2 toxin rapidly disappears from the plasma of animals experimentally administered this toxin, with mean plasma depletion half-lives of

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13.8, 17.4, and 5.3 minutes in swine, cattle, and dogs, respectively (Beasley et al., 1986; Sintov et al., 1987). Diacetoxyscirpenol is even more rapidly cleared with half-lives of 11.6 minutes in swine and 6.4 minutes in cattle (Coppock et al., 1987). These short half-lives are primarily due to rapid metabolism by up to four competing biotransformation pathways including: ester hydrolysis (Bauer et al., 1985; Knupp et al., 1987; Corley et al., 1986; Pace et al., 1985; Visconti et al., 1985), conjugation with glucuronic acid (Gareiss et al., 1986; Corley et al., 1985; Côté et al., 1986), hydroxylation (Corley et al., 1985; Knupp et al., 1985; Yoshizawa et al., 1985), and epoxide reduction or deepoxidation (Chatterjee et al., 1986; Sakamoto et al., 1986; Côté et al., 1986; Yoshizawa et al., 1983,1985). Recently, we demonstrated that anaerobic bovine rumen microflora can reduce the epoxide group of the trichothecenes T-2 toxin, DAS, and DON (Swanson et al., 1987a). Deepoxy metabolites of T-2 and DAS were isolated and demonstrated to be nontoxic to brine shrimp (Swanson et al., 1987b). In the present study, intestinal microflora from six species were investigated for their role in deepoxidation of trichothecenes. In addition, deepoxy T-2 was synthesized and its potential for dermal irritation evaluated in the rat skin bioassay.

Experimental

Chemicals

Standards of T-2 toxin and diacetoxyscirpenol (DAS) were produced in our laboratory from cultures of Fusarium sporotrichioides. Metabolites were prepared as previously described (Swanson et al., 1987a,b). The purity of all standard compounds was greater than 98% as determined by capillary gas chromatography of the corresponding trimethylsilyl ether derivatives and flame ionization detection. Structures of T-2, DAS, and their metabolites are shown in Figure II.5.

Animals

Male 200- to 250-gram Sprague-Dawley rats and male 20- to 25-gram Balb/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Feces from all other species were obtained from healthy control animals housed at the College of Veterinary Medicine, University of Illinois.

Preparation of Inoculum

Fresh feces (feces collected within 5 minutes after defecation) were collected in plastic bags, immediately flushed with dry nitrogen and processed within 15 minutes. A 2-ml aliquot of a 1:5 suspension of feces and glucose-yeast extract (GYE) medium was used as inoculum. Intestinal microflora were obtained from the cecum of rats anesthetized with ether. The cecum was removed through a small ventral incision, and the contents were removed and rapidly transferred to GYE media as described for feces.

Culture Conditions

All media were prepared and transfers conducted under an oxygen-free nitrogen atmosphere. Test tubes containing 1 mg of toxin dissolved in 8 ml of GYE were inoculated with 2 ml of fecal or intestinal suspensions and incubated for 4 days at 37°C.

Extraction and Analysis of Metabolites

Two ml aliquots of the incubation mixtures were added to 1 g C18 cartridges (Analytichem International, Harbor City, CA) preconditioned with 5 ml methanol followed by 5 ml of water. The cartridges were rinsed with water (1 ml) and the toxins eluted with 2 ml of methanol. The eluate was concentrated to near dryness and the residue redissolved in 0.8 ml of ethyl acetate and transferred to 0.65 g column of Florisil packed in ethyl acetate. An additional 6 ml of ethyl acetate was added to the top of the column. The total eluate was collected, concentrated to dryness over nitrogen, and the residue redissolved in ethanol. Aliquots were removed and concentrated to dryness for gas chromatographic analysis.

Derivatization and Gas Chromatography

Formation of the corresponding trimethylsilyl (TMS) ether derivatives was accomplished by adding 50 µl of TMS reagent (BSTFA + TMCS + TSIM; 11:2:3) to the concentrated extracts and heating for 10 minutes at 60°C. The solution was then diluted with 50 µl of ethyl acetate containing 0.50 mg/ml triacontane ($C_{30}H_{62}$) as an internal standard. Capillary gas chromatography was performed on a Hewlett Packard 5790A gas chromatograph equipped with a flame ionization detector and a J & W DB 1701 fused silica capillary column (30 m x 0.25 mm id, 0.25 µ film thickness, J & W Scientific, Folsom, CA). The column oven was temperature programmed from 250°C to 275°C (hold 5 minutes) at 5°C/minute. Other conditions were: injector, 275°C; detector, 300°C; hydrogen carrier gas linear velocity, 50 cm/second. One µl of sample was injected using a split injection with a split ratio of 50:1.

Synthesis of Deepoxy T-2

Deepoxy T-2 was prepared using a modification of the method described by Colvin and Cameron (1986) for the preparation of deepoxy triacetoxyscirpenol. T-2 toxin (0.25 mmol) was dissolved in dry tetrahydrofuran (15 ml). The toxin was cooled in a dry ice/isopropanol bath and four equivalents of tungsten hexachloride and 12 equivalents of n-butyl lithium (in hexane) were slowly added under an argon atmosphere. After refluxing the mixture for 6 hours, the solvent was removed in vacuo on a rotary evaporator. The residue was redissolved in ethyl acetate and washed with 2 N sodium hydroxide. The organic layer was washed with water and dried over anhydrous sodium sulfate. Purification of the resulting deepoxy T-2 was accomplished by HPLC with a reverse phase column (Alltech C18, 10 mm id x 25 cm, 10 µ) in 55% methanol at a flow rate of 2 ml/minute. Fractions were monitored by silica thin-layer chromatography with chloroform-methanol (9 + 1) as the mobile phase. Compounds were visualized after charring with methanolic sulfuric acid. Appropriate

fractions were combined, concentrated and the resulting purified deepoxy T-2 recrystallized in ethyl acetate/hexane.

Nuclear Magnetic Resonance and Mass Spectrometry

Gas chromatography/mass spectrometry was performed on an Extranuclear Simulscan 300 series mass spectrometer using methane positive chemical ionization. Proton NMR spectra were obtained in $CDCl_3$ on a General Electric QE-300 spectrometer operating at 300 MHz.

Toxicity Determinations

A 5 x 3 cm patch of hair on the back of five male rats was clipped and the area marked into six separate sections. Treatments were as follows: 1) T-2 toxin (40 ng/µl), 2) T-2 toxin (160 ng/µl), 3) DE T-2 (160 ng/µl), 4) DE T-2 (1,600 ng/µl), 5) DE T-2 (16,000 ng/µl), and 6) ethyl acetate vehicle control. Test compounds were dissolved in ethyl acetate and 3 µl of each of the six test solutions were applied to the skin of each rat. Dermal reaction to the test solutions was observed 48 hours after toxin administration and given scores ranging from 0 (no reaction) to 4 (severe reaction) for both edema and erythema as described by Hayes and Schiefer (1979).

Rats were killed 48 hours after toxin administration by barbituate overdose and the treated skin was removed and fixed in 10% neutral buffered formalin. The fixed tissue was routinely processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for histopathologic examination.

For acute toxicity determinations, seven mice per group were administered the test compounds dissolved in 0.5 ml of corn oil by intraperitoneal injection. The four treatment groups were: 1) DE T-2 (12 mg/kg), 2) DE T-2 (60 mg/kg), 3) T-2 toxin (12 mg/kg), and 4) vehicle control (corn oil only). Animals were observed for 5 days after dosing.

Results

Biotransformation of DAS by Fecal Microflora

Fecal microflora from horses, cattle, dogs, swine, rats, and chickens was compared by incubating DAS with mixed bacterial suspensions under anaerobic conditions for 4 days. Percentage conversion of DAS to total deepoxy metabolites by the six species is displayed in Figure II.6. No deepoxy compounds were detected upon incubation of DAS with microflora from horses, dogs, or chickens, whereas fecal microflora from rats, swine, and cattle converted DAS predominantly to deepoxy products. Microflora from rats were the most efficient at reducing the epoxide group; all of the parent compound was converted to deepoxy metabolites. No deepoxy metabolites were formed upon incubation of DAS with rat fecal microflora under aerobic conditions (data not shown).

The molar percentages of individual metabolites recovered from the fecal fermentations are shown in Table II.1. Microflora from all six species displayed varying degrees of esterase activity, however deepoxidation products were observed only with microflora obtained

from cattle, swine, and rats. The major products of incubations with rat, swine, and cattle fecal microorganisms were DE MAS and DE SCP. By contrast, the major products of horse, dog, and chicken microflora incubations were the simple C-4 deacylated product, MAS, in addition to unmetabolized parent DAS.

Biotransformation of T-2 Toxin by Cecal Microflora

T-2 toxin was completely biotransformed by rat cecal microflora, predominantly to C-4 and C-15 deacylated, deepoxy products (Table II.2). The major product was the result of epoxide reduction and C-4 deacetylation which yielded the compound DE HT-2, although minor quantities of DE TRIOL were also detected. No products resulting from C-8 ester hydrolysis (TOL, 4-deacetylneosolaniol or their deepoxy analogs) were detected in any of the T-2 toxin incubations. As with T-2 toxin, diacetoxyscirpenol was also completely biotransformed by the cecal microflora to yield DE MAS and DE SCP. In addition, traces of SCP (less than 0.5%) were also found.

The polar trichothecenes, T-2 tetraol and scirpentriol, were incubated with rat intestinal microflora to determine if ester side groups and lipophilicity were important factors in epoxide reduction. The majority of both polar trichothecenes were reduced by the suspensions of anaerobic rat intestinal microflora to yield their corresponding deepoxy analogs, DE TOL and DE SCP (Table II.2).

Synthesis of DE T-2

Deepoxy T-2 was obtained in a 35% overall yield. Final purity was greater than 99% as determined by capillary gas chromatography of the corresponding TMS ether derivative. The positive chemical ionization mass spectrum of the TMS derivative is shown in Figure II.7. The pseudomolecular ion at m/z 523 establishes the molecular weight at 522. The major fragments at m/z 507, 463, 421, 361, and 301 were 16 mass units less than the epoxy congener, T-2 toxin. The proton NMR spectrum of DE T-2 is shown in Figure II.8. Instead of doublet resonances at 2.81 and 3.05 ppm due to the C-13 methylene protons of the epoxide group in T-2 toxin, singlet resonances were observed at 4.77 and 5.11 ppm in DE T-2. These singlets were assigned to the terminal vinyl protons at the C-13 position. These data supported the identification of the structure of the synthesized product as deepoxy T-2 (3α-hydroxy-4ß,15-diacetoxy-8α-[3-methylbutyryloxy]-trichothece-9,12-diene).

Dermal Irritantion of Deepoxy T-2

The dermal irritant toxicity of DE T-2 and T-2 toxin was compared in the rat skin bioassay and results are shown in Table 3. Erythema was the most consistant response observed. Edema was only observed at the highest T-2 toxin dose (160 ng/µl). Dermal irritation by DE T-2 was observed only at a concentration of 16,000 ng/µl, the highest concentration examined. At this concentration, the dermal response produced by DE T-2 was equivalent to that caused by 40 ng/µl of T-2 toxin ($P < 0.05$).

Histologically, T-2 toxin at 160 ng/µl caused moderate fibrino-suppurative exudation with local epidermal ulceration. The intact

epidermis was moderately thickened and hyperplastic. Moderate edema and infiltration by inflammatory cells, primarily neutrophils, was present in the epidermis and dermis. At 40 ng/µl, similar but less severe lesions were present; no epidermal ulceration was noted. DE T-2 induced histologic lesions only at 16,000 ng/µl, and the lesions were milder than those induced by T-2 toxin at 40 ng/µl. Only mild epidermal hyperplasia and minimal epidermal/dermal inflammation was observed.

Intraperitoneal Toxicity of Deepoxy T-2

No lethality was observed in mice administered DE T-2 at either dosage. In addition, no clinical signs of toxicity were seen in any mice administered DE T-2. Five of seven mice administered 12 mg/kg T-2 toxin died within 4 days postdosing. The remaining two mice survived but displayed clinical signs consistant with T-2 intoxication including: rough hair coats, diarrhea, decreased startle response, and lethargy.

Discussion

As in previous studies with bovine rumen microflora (Swanson et al., 1987a), direct deepoxidation products (i.e., DE T-2 or DE DAS) were not detected upon incubation with fecal microflora from any of the six species nor after incubation of either T-2 toxin or DAS with rat cecal intestinal microflora. Only C-4 and C-15 deacylated, deepoxy products were observed. The lack of epoxide reduction by rat cecal microflora or rat fecal microflora under aerobic conditions confirms that the microorganism(s) involved in this reaction are anaerobes.

The rat cecal microflora were not able to hydrolyze the C-8 ester position in T-2 toxin to yield TOL or its deepoxy analog, DE TOL. T-2 tetraol and DE TOL are major metabolites detected in the urine and feces of cattle, guinea pigs, and rats orally administered T-2 toxin (Chatterjee et al., 1986; Pace et al., 1985; Pfeiffer, Swanson, and Buck, submitted for publication). The lack of T-2 toxin C-8 ester hydrolysis by rat cecal microflora in this study confirms that products of ester cleavage at this position observed in whole animal studies are solely the result of mammalian metabolism by carboxyesterases and not microflora inhabiting the gut.

The polar trichothecenes TOL and SCP were extensively biotransformed to their deepoxy analogs, DE TOL and DE SCP. Both TOL and SCP lack ester side groups and are water soluble polar metabolites. The fact that the epoxide group in these very polar trichothecenes was reduced to a carbon-carbon double bond suggests ester side groups and lipid solubility are not important factors in deepoxidation reactions by anaerobic intestinal microflora.

The species variations in conversion of trichothecenes to deepoxy products as observed in this in vitro study correlates with previously published whole animal studies, if experiments utilizing thin-layer chromatography as a separation technique are excluded. Published studies utilizing TLC as the means for separation generally omit any reference to deepoxidation products, regardless of the specific trichothecene mentioned. It is unlikely that deepoxy metabolites would be detected by thin-layer chromatographic

techniques, even if these metabolites were present in the samples, for the following reasons: 1) deepoxy standards of trichothecenes are not readily available, 2) deepoxy trichothecenes separate poorly from their epoxy analogs by normal phase thin-layer chromatography, and 3) deepoxy trichothecenes do not react with p-nitrobenzylpyridine to give characteristic blue color observed with epoxytrichothecenes (Sakamoto et al., 1986; Swanson et al., 1987a,b). Several studies have demonstrated that rats, cattle, and swine administered trichothecene mycotoxins experimentally excrete a significant portion of the dose as deepoxy metabolites. Rats administered DAS orally excrete DE MAS and DE SCP as the major products (Sakamoto et al., 1986). Rats given T-2 toxin orally (Pfeiffer, Swanson, and Buck, submitted for publication) excrete a variety of deepoxy metabolites. The first deepoxy trichothecene animal metabolite detected, called DOM-1, was originally identified in the feces and urine of rats orally administered DON (Yoshizawa et al., 1983). Recently, Lake et al. (1987) confirmed that deepoxy DON (DOM-1) is the major free metabolite eliminated by rats orally administered C-14 radiolabelled DON. They observed 10% of the radioactivity in the urine and 13% in the feces was excreted as the deepoxy metabolite of DON.

Cattle orally administered either T-2 toxin or DON excrete deepoxy metabolites as major excretion products in both the feces and urine (Côté et al., 1986; Chaterjee et al., 1986). Previous work demonstrated that rumen microflora have the capacity to reduce the epoxide group of several trichothecenes (Swanson et al., 1987a; King et al., 1984). In this study, we established that epoxide reduction can occur not only through the action of anaerobic bovine rumen microflora but also as a result of biotransformation by anaerobic gastrointestinal microorganisms. Deepoxidation by both rumen microflora and intestinal microorgansims may account for the decreased sensitivity to trichothecene toxicoses by ruminants species. Although swine fecal microflora yielded predominantly deepoxy metabolites of T-2 toxin in the present study, only minor quantities were detected in the gastrointestinal contents and tissues of swine administered T-2 toxin intravascularly (Corley et al., 1985).

In this study, microflora from chickens, dogs, and horses did not reduce the epoxide group of DAS. Although whole animal disposition data for trichothecenes is limited for dogs (Sintov et al., 1986) and entirely lacking for horses, several studies have been conducted in poultry with both T-2 toxin and DON (Kubena et al., 1985; Visconti et al., 1985). In these in vivo poultry studies, trichothecene residues in tissues and excreta were detected by either gas chromatography or GC-MS techniques, methods which are capable of resolving deepoxy trichothecene metabolites from their epoxy analogs. However, no deepoxy compounds were detected in any of the tissue or excreta samples analyzed, supporting the in vitro evidence presented here that chickens lack the necessary microflora for epoxide reduction.

T-2 toxin and diacetoxyscirpenol are both potent dermal irritants and dermal irritation has been a useful bioassay in detection of these trichothecenes (Shiefer and Hayes, 1979). Histopathologic examination of the skin following dermal application of the toxins was more sensitive than gross evaluation. Histologically, 16,000 ng/µl DE T-2 was less toxic than 40 ng/µl T-2 toxin. Thus, deepoxy T-2 was 400-fold less toxic than T-2 toxin in the rat dermal

irritation bioassay. No lethality was observed after IP admistration of DE T-2 to mice at dosages of 60 mg/kg, a dosage approximately six times the LD_{50} of T-2 toxin. In addition, no adverse clinical signs were noted in any of the mice administered DE T-2.

The toxicity of DE T-2 has not previously been studied, although we recently demonstrated that the metabolites DE HT-2 and DE MAS were at least 50-fold less toxic to brine shrimp than their corresponding epoxy analogs, HT-2 and MAS (Swanson et al., 1987b). These data confirm that the epoxide group in trichothecene mycotoxins is essential for toxicity and demonstrate that reduction of the epoxide group by anaerobic intestinal microflora is an effective single-step detoxification reaction.

The dramatic decrease in toxicity following reduction of the epoxide group in T-2 toxin suggests that species with intestinal microflora containing high deepoxidation activity (rats, swine, and cattle) should display reduced sensitivity to the trichothecenes, compared to species lacking microflora with deepoxidation activity (horses, chickens, and dogs). This relationship, however, is not supported by existing acute toxicity data (Table 4). The oral LD_{50} of T-2 toxin in rats (a species displaying high deepoxidation activity) is not noticably different than that of chickens (a species displaying no deepoxidation activity). The LD_{50} of T-2 toxin in swine is the lowest of all species examined, yet in the present study swine fecal microflora displayed relatively high deepoxidation activity with DAS. If deepoxidation were the sole pathway of biotransformation involved, one would expect species with microflora capable of deepoxidation to be significantly less sensitive to the trichothecene toxins and display correspondingly higher LD_{50} values. The metabolism of trichothecenes in whole animals is very complex, involving up to four different competing pathways. Also, enterohepatic recirculation may play an important role in determining toxicity. Additional studies should be done to determine the role deepoxidation plays in the overall metabolism and toxicity of trichothecenes *in vivo*.

Although no relationship between acute lethal toxicity data and *in vitro* deepoxidation activity was noted among species, it is unrealistic to assume epoxide reduction by gastrointestinal microflora is unimportant in the overall toxicity of trichothecenes within a given species. Treatments such as antibiotic therapy may reduce microflora in the gastrointestinal tract and decrease the rate of epoxide reduction, a detoxification reaction. The overall result of reducing this detoxification reaction would be to delay elimination of the parent compound and/or toxic epoxy metabolites, thereby increasing toxicity. Experiments addressing these questions are in progress.

Acknowledgement

The authors thank P. Sanders for the mass spectral analysis. This work was supported in part by the U.S. Army Medical Research Development Command Contract No. DAMD-17-85-C-5224.

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Table II.1 *In vitro* biotransformation of DAS by anaerobic fecal microorganisms from six species

Species	% Recovered (Mean ± SEM)[a]				
	DAS	MAS	SCP	DE MAS	DE SCP
Rat	ND[b]	ND	ND	66.5 ± 6.2	33.5 ± 6.2
Swine	ND	12.6 ± 7.5	2.2 ± 1.1	61.7 ± 5.9	23.5 ± 3.1
Cow	7.9 ± 2.4	15.8 ± 11.3	3.7 ± 2.4	32.4 ± 13.0	40.1 ± 16.1
Dog	11.2 ± 1.4	88.7 ± 1.2	0.2 ± 0.2	ND	ND
Horse	36.1 ± 6.8	60.1 ± 5.9	3.8 ± 3.9	ND	ND
Chicken	43.5 ± 13.7	48.6 ± 8.8	7.9 ± 5.2	ND	ND

[a]Molar percent of total metabolites recovered.
[b]ND = none detected
Number of animals = rat, 4; swine, 4; cattle, 6; dog, 3; horse, 3; chicken, 4.

Table II.2 *In vitro* biotransformation of T-2 toxin, diacetoxyscirpenol, T-2 tetraol, and scirpentriol by anaerobic rat cecal microorganisms

Compound Added	Metabolite	% Recovered[a]
T-2	T-2	ND
	HT-2	ND
	TRIOL	ND
	DE HT-2	97.6 ± 1.2
	DE TRIOL	2.4 ± 1.2
DAS	DAS	ND
	MAS	ND
	SCP	0.3 ± 0.2
	DE MAS	81.9 ± 1.6
	DE SCP	17.8 ± 1.7
TOL	TOL	12.7 ± 2.4
	DE TOL	87.3 ± 2.3
SCP	SCP	4.3 ± 2.2
	DE SCP	95.7 ± 2.3

[a]Mean percent of total metabolites recovered ± SEM (three animals).
ND = none detected.

Table II.3 Dermal irritation of T-2 toxin and its deepoxy analog in rats

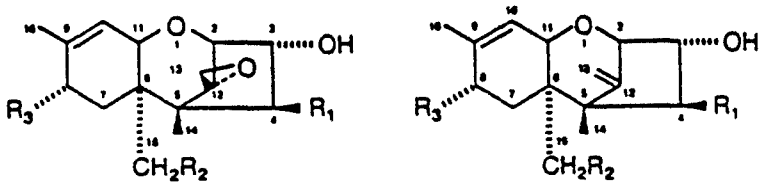
Compound	Concentration[a]	Score[b]
Control	0	0 ± 0
T-2	160	2.10 ± 0.22
T-2	40	0.90 ± 0.55
DE T-2	160	0 ± 0
DE T-2	1,600	0 ± 0
DE T-2	16,000	0.70 ± 0.67

[a]Concentration of test compound in ng/μl.
[b]Dermal irritation score for erythema; mean ± SD (N = 5).
0 = no reaction, 4 = severe erythema.

Table II.4 Comparison of T-2 toxin acute toxicity in various animal species

Species	Route	LD_{50}	Reference
Rat	Oral	4.0	Marasas et al. (1969)
		5.2	Sato and Ueno (1977)
Mouse	Oral	10.5	Sato and Ueno (1977)
	IP	5.2	Sato and Ueno (1977)
		9.1	Thompson and Wannemacher (1986)
	SC	3.3	Thompson and Wannemacher (1986)
Chicken	Oral	4.0	Hoerr et al. (1981)
		3.6	Sat and Ueno (1977)
		4.9	Chi et al. (1978)
Guinea pig	Oral	3.1	DeNicola et al. (1978)
Swine	IV	1.2	Weaver et al. (1978)

Figure II.5 Structure of T-2 toxin, diacetoxyscirpenol, and their metabolites formed in vitro under anaerobic incubation conditions.



A B

Compound	Skeleton	R1	R2	R3
DAS	A	OAC	OAC	H
15-MAS	A	OH	OAC	H
SCP	A	OH	OH	OH
DE MAS	B	OH	OAC	H
DE SCP	B	OH	OH	H
T-2	A	OAC	OAC	ISV
HT-2	A	OH	OAC	ISV
TRIOL	A	OH	OH	ISV
TOL	A	OH	OH	OH
DE T-2	B	OAC	OAC	ISV
DE HT-2	B	OH	OAC	ISV
DE TRIOL	B	OH	OH	ISV
DE TOL	B	OH	OH	OH

ISV = $OCOCH_2CH(CH_3)_2$, OAC = $OCOCH_3$, DAS = diacetoxyscirpenol, MAS = 15-monoacetoxyscirpenol, SCP = scirpentriol, DE MAS = deepoxy monoacetoxyscirpenol, DE SCP = deepoxy scirpentriol, TRIOL = T-2 triol, TOL = T-2 tetraol, DE T-2 = deepoxy T-2, DE HT-2 = deepoxy HT-2, DE TRIOL = deepoxy T-2 triol, DE TOL = deepoxy T-2 tetraol.

Figure II.6 Conversion of diacetoxyscirpenol (DAS) to total deepoxy metabolites by fecal microflora from six species.

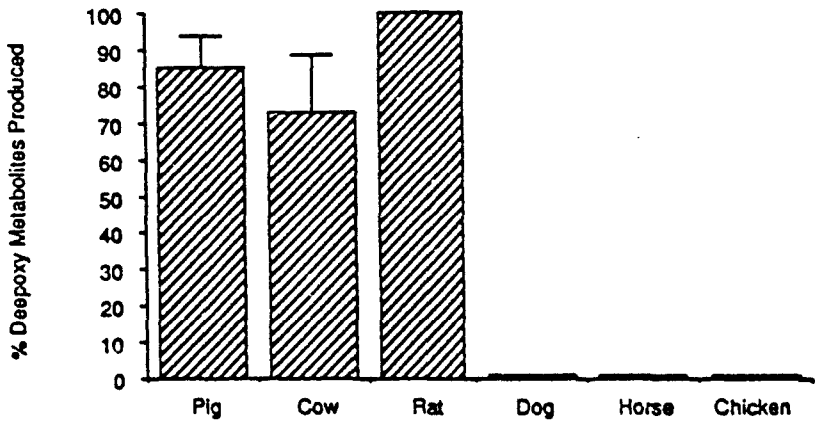


Figure II.7 Positive chemical ionization mass spectrum of deepoxy T-2 TMS derivative.

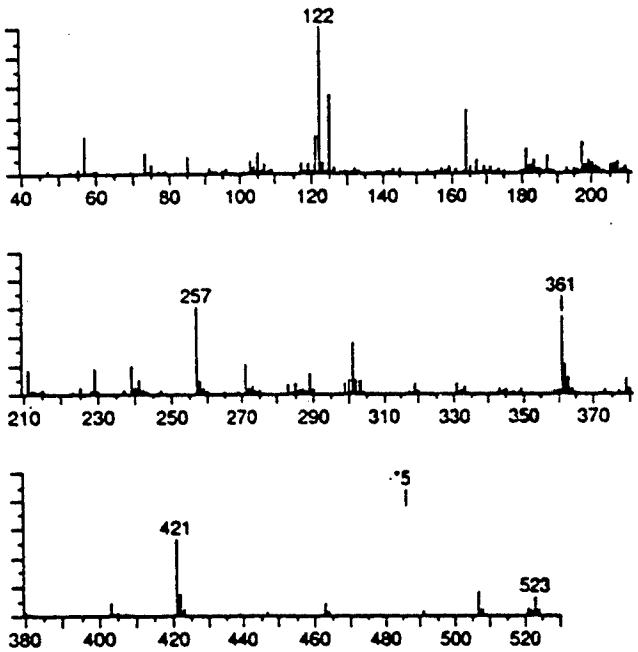
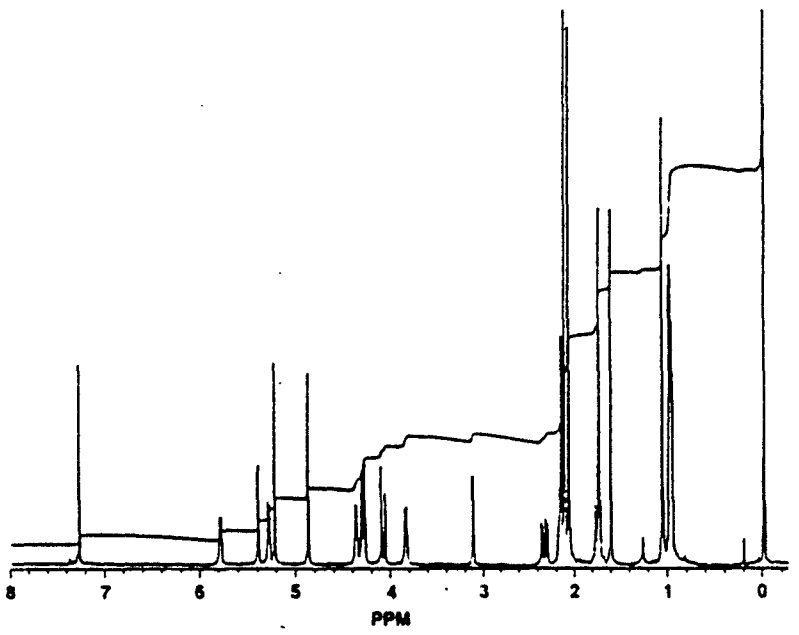


Figure II.8 Proton NMR of deepoxy T-2.



b. Isolation and characterization of 4'-hydroxy T-2 toxin, a new metabolite of the trichothecene mycotoxin T-2

by
C. A. Knupp, D. G. Corley, M. S. Tempesta, and S. P. Swanson.

Abstract

Biotransformation of the trichothecene mycotoxin T-2 by the hepatic S-9 fraction prepared from phenobarbital-treated rats yielded a new metabolic product designated RLM-3. The metabolite was purified from the hepatic preparation using preparative HPLC. The structural analysis of RLM-3 was carried out using gs chromatography/mass spectometry and proton and carbon-13 NMR. RLM-3 was identified as 4'-hydroxy T-2. THe toxicity of RLM-3 in comparison to T-2 toxin and 3'-hydroxy T-2 was assessed using the rat skin bioassay technique. THe metabolite 4'-hydroxy T-2 was shown to be deacylated at the C-4 position to yield 4'-hydroxy HT-2 when incubated with rat hepatic S-9 preparations.

Introduction

T-2 toxin [4,15-diacetoxy-8-(3-methylbutyryloxy)-3-hydroxy-12,13-epoxytrichothec-9-ene] is a naturally occurring toxic fungal metabolite produced by various species of Fusaria (Bamburg and Strong, 1971). It is rapidly biotransformed in a variety of animal species by several pathways including hydrolysis, hydoxylation, deepoxidation and conjugation, to yield many different metabolites. Oxidation of the C-3' position on the isovaleryl side chain to yield the metabolites 3'-hydroxy T-2 and 3'-hydroxy HT-2 has been described in chickens, rats, mice, and swine (Visconti and Mirocha, 1985; Yoshizawa et al., 1982; Yoshizawa et al., 1984; Corley et al., 1985; Knupp et al., 1986). Another hydroxylated metabolite, designated TC-6, excreted in the feces and urine of a cow orally dosed with T-2 (Pawlosky and Mirocha, 1984) was tentatively identified as 3'-hydroxy-7-hydroxy-HT-2 toxin by Pawlosky and Mirocha (1984). Recently, we have reported on a new hydroxylated T-2 metabolite, designated RLM-3, produced in vitro by hepatic microsomes prepared from rats, mice, and chickens (Bamburg and Strong, 1971). This compound has now been identified as 4'-hydroxy T-2 toxin [4ß,15-diacetoxy-3α-hydroxy-8α-(3-methyl-4-hydroxybutyryl-oxy)-12,13-epoxytrichothec-9-ene]. This paper reports on the production, isolation, purification, and identification of the metabolite RLM-3. A preliminary study in rats comparing the dermal toxicity of Rlm-3 with that of T-2 toxin and 3'-hydroxy T-2 was also conducted.

Materials and Methods

Animal Treatment and Hepatic S-9 Preparation

Male Sprague-Dawley rats weighing 400 to 500 g were given 0.1% phenobarbital in their drinking water for 5 days prior to preparation of the hepatic S-9 fraction. Following euthanasia, the livers were removed and homogenized in three volumes of ice-cold 0.25 M sucrose. The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant, referred to as S-9, filtered through glass wool.

Metabolite Production and Extraction

In a 1 L erlenmeyer flask, 50 ml of S-9 was mixed with 350 ml Tris buffer (0.05 M, pH 7.4 at 37°C) containing 0.15 M KCl. Paraoxon (5.75 µmol, dissolved in 0.25 ml ethanol) was added, followed by 0.33 mmol NADP, 1.67 mmol glucose-6-phosphate, and 5.00 mmol MgCl2. The incubation mixture was agitated for 3 to 5 minutes, then 0.134 mmol T-2 toxin (98% + pure) dissolved in 0.5 ml of ethanol was added. The reaction was carried out at 37°C for 3 hours in a shaking water bath. At the end of the incubation period, 100 g of NaCl followed by 130 ml of acetone was added to precipitate the hepatic proteins. The solution was filtered through a buchner funnel packed with celite and the funnel rinsed with 100 ml ethyl acetate. The aqueous fraction was extracted three times with ethyl acetate. The ethyl acetate fraction was dried over sodium sulfate, transferred to a boiling flask and concentrated to dryness. The resulting oil was transferred to a glass vial for further purification.

Metabolite Purification

The oil product ws dissolved in chloroform (pentene preserved) and chromatographed on a 25 g Florisil column (60 to 100 mesh) packed in chloroform. The column was rinsed with 250 ml chloroform, followed by 150 ml chloroform:acetone (95:5, v/v). The metabolite RLM-3 was eluted with 250 ml chloroform:methanol (95:5, v/v). The eluate was concentrated to dryness on a rotary evaporator and the resulting oil transferred to a glass vial for HPLC purification. A Perkin-Elmer Series 4 liquid chromatograph equipped with an Alltech cyano column (25 cm x 10 mm id x 10 µm) was programmed to run a linear gradient starting from chloroform:hexane (4:1, v/v) to chloroform:hexane: acetone (65:20:15, v/v) over a 110-minute time span at a flow rate of 1.5 ml/minute. Aliquots of selected fractions were spotted on precoated silica gel thin layer chromatography plates (20 x 20 cm, 0.25 mm gel thickness; from Whatman). The plates were developed in chloroform:methanol (9:1, v/v) and air-dried. The compounds were visualized under long-wave ultraviolet light after charring with 30% sulfuric acid in methanol at 110°C for 3 to 5 minutes. These normal phase HPLC chromatographic connditions provided adequate separation of 3'hydroxy T-2 from the later eluting RLM-3, but RLM-3 did not separate from neosolaniol (NEO). For this reason, the fractions containing RLM-3 and NEO were combined, evaporated to dryness, and redissolved in methanol:water (2:3, V/V) for reverse phase HPLC. A Perkin-Elmer Series 4 liquid chromatograph equipped with an Alltech C18 column (25 cm x 10 mm id x 10 µm) was programmed with a linear gradient from methanol:water (2:3, v/v, initial hold of 20 minutes) to 100% methanol over a 40-minute time period at a flow rate of 2.0 ml/minute. By reverse phase HPLC, NEO eluted prior to RLM-3 and complete separation of these two compounds was achieved. However, RLM-3 did not resolve from 3'-hydroxy T-2 using reverse phase HPLC. Fractions containing RLM-3 were combined and concentrated to dryness. RLM-3 was recrystallized by dissolving the oil in ethyl acetate, then adding drop-wise into cold hexane.

Metabolism of RLM-3

The metabolism of RLM-3 was investigated using the hepatic S-9 fraction (9,000 x g supernatant of a liver homogenate) prepared from

control rats (no phenobarbital pretreatment). To triplicate 20 ml glass vials, 0.33 ml of S-9 was mixed with 2.7 ml of Tris buffer (as described above) containing 1.8 mol NADP, 9.0 mol glucose-6-phosphate and 27.0 mol magnesium chloride. RLM-3 (0.250 mg dissolved in 0.025 ml ethanol) was added and the mixture agitated at 37°C for 1 hour in a shaking water bath. At the end of the incubation period, the sample was applied to a 500 mg C18 cartridge (J. T. Baker; preconditioned with methanol and water). The cartridge was rinsed with water (2 x 1 ml) and the metabolites eluted with methanol (2 x 1 ml) using a Analytichem Vac Elute vacuum manifold system (Harbor City, CA). The solvent evaporated under nitrogen and the residue redissolved in ethanol. An aliquot was removed and concentrated to dryness for gas chromatographic analysis.

Dermal Toxicity of RLM-3 in Rats

The backs of five male Sprague-Dawley rats weighing 350 tp 400 g were clipped and 5 L of each test solution was applied to one of six dermal application sites on each rat with a syringe. The total amount of toxin applied to each site was: T-2 toxin at 0.11, 0.21, and 0.54 nmol per site; 3'-hydroxy T-2 at 1.04 nmole; and RLM-3 at 1.04 nmole. Each toxin was dissolved in ethyl acetate and an area treated with ethyl acetate alone served as a control. At 48 hours postapplication the results were evaluated and the severity of the reaction scored based on a scale from one to four. A score of one indicated a mild reaction (slight reddening of the skin), while a score of four indicated a severe reaction (severe erythema with evidence of exudation, scaling and petechiation).

Derivatization and Gas Chromatography

RLM-3 was analyzed both as the trimethylsilyl (TMS) and trifluoroacyl (TFA) derivatives. The silylated derivative was formed by dissolving the sample in 0.04 ml of ethyl acetate-isooctane (1 + 1, v/v) containing 0.25 mg/ml triacontane as an internal standard, then adding 0.01 ml bis trimethylsilyl) trifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS):N-trimethylsilylimidazole (TSIM) (11:2:3, v/v/v; Pierce Chemical Co., Rockford, IL) and heating at 60°C for 10 minutes. The trifluoroacyl derivative was formed by adding 0.05 ml trifluoroacetic anhydride (TFAA, Pierce Chemical Co., Rockford, IL) and heating at 60°C for 20 minutes. The excess TFAA was then evaporated under a gentle stream of nitrogen and 0.05 ml toluene was added as the carrier solvent. Gas chromatography was performed on a Hewlett Packard 5790A gas chromatograph equipped with a flame ionization detector (FID) and a 30 meter length x 0.25 mm id and a 0.25 µ film thickness J & W DB 170i fused silica capillary column, using a split injection mode (split ratio of 40:1). Operating conditions were as follows: injector, 275°C and detector, 300°C. Hydrogen was used as the carrier gas at a linear velocit of 45 cm/second. The oven temperature was temperature programmed from 250 to 275°C at a rate of 5°C/minute for the TMS derivatives and from 225 to 275°C at a rate of 5°C/minute for the TFA derivatives.

Mass Spectrometry

Gas chromatography-mass specrometry was performed on an Extranuclear Simulscan 300 Series quadrapole mass spectrometer with a Perkin Elmer

Series 2 gas chromatograph equipped with a J & W DB 1701 capillary column. Positive methane chemical ionization (CI) spectra of both the TMS and TFA derivatives of RLM-3 and its S-9 biotransformation product MR-1 were obtained, in addition to electron impact spectra of the TMS derivatives. The electron energy was set at 70 ev, the interface temperature at 270°C and the source temperature at 100°C.

Nuclear Magnetic Resonance Spectra

The NMR experiments were performed on a Nicolet NT-300 WB spectrometer equipped with 5-mm 1H and 13C probes operating at 300.05 and 75.45 MHz, respectively. The sample was run in CDCl3 and referenced to internal TMS (0.0 ppm) for proton NMR and to CDCl3 (77.0 ppm) for carbon spectra. The distortionless Enhancement by Polarixation Transfer (DEPT) experiment used the pulse sequence of Doddrell et al. (1982).

Results and Discussion

The chemical structures and resolution of T-2 toxin and its derivatives by thin-layer chromatography and capillary gas chromatography are given in Table II.5. Since RLM-3 was difficult to resolve from the neosolaniol also present in the preparation using normal phase HPLC, it was necassary to use reverse phase HPLC to achieve complete separation. However, reverse phase HPLC did not resolve RLM-3 from 3'-hydroxy T-2. Therefore, both normal and reverse phase HPLC were required in order to obtain purified RLM-3 (> 99% pure by capillary gas chromatography). Purification of RLM-3 was further complicated since this compound hydrolyzed readily to yield neosolaniol, especially under aqueous conditions.

RLM-3 was a minor metabolite of T-2 toxin in the rat hepatic S-9 preparations (5-10% of the added substrate), while 3'-hydroxy T-2 was the major metabolic product (greater than 85%). Treatment of rats with phenobarbital to induce mixed function oxidase activity was necassary to increase the yield of the hydroxylated metabolites of T-2 for preparative isolations. Paraoxon, a potent esterase inhibitor, was added to the incubation mixtures to prevent hydrolysis of the oxidized products by carboxyesterases. Although RLM-3 was formed in the rat hepatic preparations at only 5 to 10% of the added T-2 toxin as much as 15% RLM-3 was observed in liver S-9 preparations from phenobarbital treated chickens (data not shown). Hepatic preparation from rats used to produce sufficent quantities of RLM-3 for structural identification and preliminary toxicological evaluations because rats and appropriate housing were readily available. The hepatic S-9 fraction rather than a microsomal preparation was chosen for production purposes for two reasons: 1) isolation of the S-9 fraction did not require an ultracentrifugation step and 2) lower molar amounts of the components required in the NADPH generating system were needed with the S-9 fraction due to the presence of endogenous cofactors.

The positive CI spectrum of the TMS ether derivative of RLM-3 is shown in Figure II.9. The M + 1 pseudomolecular ion at m/z 627 and the methan dimer adduct (C_2H_5) at m/z 655 (M + 29) are both present. This establishes the molecular weight of the TMS derivative as 626, identical to that of 3'-hydroxy T-2. Since these two compounds gave

similar CI spectra as the TMS derivatives, additional spectra were obtained using the corresponding TFA derivatives. Figure II.10 shows the positive CI spectrum of the TFA derivative of RLM-3. Both the dimer (M + C_2H_5) and the trimer (M + C_3H_7) methane adducts are present at m/z 703 and 715, respectively, confirming the M + 1 pseudomolecular ion at m/z 675. The positive CI spectrum of the TFA derivative of 3'-hydroxy T-2 is given in Figure 2B. When 3'-hydroxy T-2 is reacted with TFAA, two gas chromatographically reolvable isomers with a molecular weight of 560 are formed due to the dehydration of the hydroxyl group at the C-3' position (Pawlosky et al., 1984). Unlike 3'-hydroxy T-2, RLM-3 forms a single TFA derivative that gives a mass spectrum significantly different form that of 3'-hydroxy T-2. This GC/MS data supported the conclusion that RLM-3 was an isomer of 3'hydroxy T-2 with a hydroxyl group located at the C-2' or C-4' position. The molecular weight of the TFA derivatives and the fact that RLM-3 did not form two isomers when reacted with TFAA indicated that the hydroxyl group on the isovaleryl side chain must be derivatized. The EI spectrum of the TMS derivative of RLM-3 is shown in Figure II.11. The diagnostic fragment at m/z 436 indicates the ions of the 7,9-diene structure. Unlike T-2, there is no ion at m/z 85 (isovaleryl group), supporting the theory that the isovaleryl side chain has been modified.

Table II.6 lists the ^{1}H and ^{13}C assignments of RLM-3 in CDCl3. Proton and 13C NMR data clearly establish that RLM-3 has a hydroxyl group located at the C-4' position. DEPT experiments also demonstrated that RLM-3 is a mixture of diastereomers due to the formation of a chiral center at C-3' upon hydroxylation at C-4'. Based on the mass spectral and NMR data, RLM-3 was characterized as 4'-hydroxy T-2 (4β,15-diacetoxy-3α-hydroxy-8α-[3-methyl-4-hydroxybutyryloxy]-12,13-epoxytrichothec-9-ene). The metabolism of 4'-hydroxy T-2 was investigated using the hepatic S-9 fraction prepared from control rats not treated with phenobarbital. After a 60-minute incubation period in the presence of a NADPH generating system, 40% 2% (mean standard deviation) of the added 4'-hydroxy T-2 was biotransformed to a new compound designated MR-1, in addition to traces (< 5%) of neosolaniol and 4-monoacetoxy tetraol. The positive CI spectra of the TMS and TFA derivatives of MR-1 are shown in Figure II.12. Both the TMS and TFA derivatives of MR-1 yielded single peaks by capillary gas chromatography, molecular weights of 656 and 728, respectively. The molecular weight of the TMS derivative of MR-1 is identical to 3'-hydroxy HT-2, indicating ester hydrolysis occurred at the C-2 position. The molecular weight (728) of the TFA derivative of MR-1 and the lack of isomer formation (observed with 3'hydroxy T-2) was consistent with the derivatization of the C-4' hydroxyl group, similar to RLM-3. Based upon this mass spectral data, MR-1 was tentatively identified as 4'-hydroxy HT-2.

The toxicity of 4'-hydroxy T-2 was compared with T-2 toxin and 3'-hydroxy T-2 using the rat skin irritation bioassay. 4'-hydroxy T-2 (1.04 nmol) caused the most severe reaction and was the only site scored as a 4, surpassing that caused by the highest T-2 dose (0.54 nmol). The lesions caused by the application of 1.04 nmol of the isomer 3'-hydroxy T-2 were much less severe than those caused by an equivalent dose of 4'-hydroxy T-2. No reaction was noted with either the ethyl acetate controls or 0.11 nmol T-2 in any of the five rats. Application of 0.21 nmol and 0.54 nm T-2 toxin resulted in mild

(score of 1) and moderate (score of 2) reactions, respectively. THe lesions caused by the application of 1.04 nmol 3'-hydroxy T-2 were equivalent to those seen for 0.54 nmol T-2 toxin. Dermal response in each of the five rats was highly consistant for each toxin. The results of the rat skin toxicity bioassay with RLM-3 demonstrated 4'-hydroxy T-2 was more toxic than its isomer 3'-hydroxy T-2 and nearly equal in dermal toxicity to T-2 toxin. This limited toxicity data indicates that hydroxylation of T-2 toxin at the C4' position is not a detoxification reaction. It is possible that in previously reported studies analyzing the excreta of animals exposed to T-2 toxin (10), quantitation of 3'-hydroxy T-2 toxin by reverse phase HPLC or TLC may have been overestimated, since 3'-hydroxy T-2 and 4'-hydroxy T-2 do not resove well under these conditions. A single peak identified as 3'-hydroxy T-2 may have been composed of both 3'-hydroxy T-2 (as the major component) and 4'-hydroxy T-2 (as the minor component). Since oxidation of T-2 toxin to yield 3'-hydroxy T-2 and RLM-3 does not appear to be a significant detoxification mechanism, simultaneous detection as a single compound by reverse phase HPLC may not cause any significant problems. It is also likely that any 4'-hydroxy T-2 formed *in vivo* would be further biotransformed to 4'-hydroxy HT-2, which would decrease the possibility of erroneous quantitation. No *in vivo* data pertaining to 4'-hydroxy T-2 is currently available. The compound may have been present as a minor metabolic product of T-2 toxin in previous investigations, but not detected due to the lack of a reference standard. Additional experiments to detect and monitor the fate of 4'-hydroxy T-2 and 4'-hydorxy HT-2 *in vivo* are necassary to determine the toxicological significance of these two new metabolites.

Acknowledgements

The authors thank P. Sanders for the mass spectral analysis. This work was funded by the U.S. Army Medical Research and Development Command Contract No. DAMD-17-85-5224. We are grateful to the National Science Foundation for partial support of the NMR (PCM-8115599) Facility at the University of Missouri.

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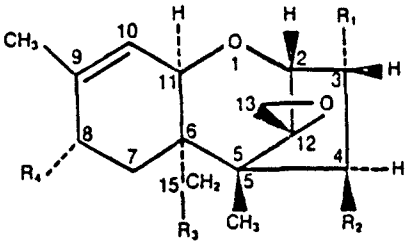
Table II.5 Chemical stuctures and resolution of T-2 toxin and its derivatives by TLC and capillary GLC

Chemical structures and resolution of T-2 toxin and its derivatives by TLC and capillary GLC



Compound	R1	R2	R3	R4	TLC R Value[a]	GLC Retention Time[b]	
						TMS	TFA
						min	
T-2 toxin	OH	OAc	OAc	X1[c]	0.72	8.09	9.78
3'-Hydroxy T-2	OH	OAc	OAc	X2[d]	0.62	11.81	10.07, 11.04[e]
RLM-3	OH	OAc	OAc	X3[f]	0.53	12.87	12.24
Neosolaniol	OH	OAc	OAc	OH	0.45	5.23	
HT-2	OH	OH	OAc	X1	0.37	7.38	6.84
3'-Hydroxy HT-2	OH	OH	OAc	X2	0.27	10.63	7.10, 7.79[e]
T-2-triol	OH	OH	OH	X1	0.22	5.84	5.54
MR-1	OH	OH	OAc	X3	0.20	11.62	9.06
T-2 tetraol	OH	OH	OH	OH	0.10	3.62	3.35

[a] On silica gel TLC plates developed in $CHCl_3$:MeOH (9:1, v/v).
[b] Retention times of TMS and TFA derivatives. See text for conditions.
[c] X1 = $OCOCH_2CH(CH_3)_2$.
[d] X2 = $OCOCH_2C(OH)(CH_3)_2$.
[e] The 3'-hydroxy metabolites form two isomers when derivatized with TFAA (8).
[f] X3 = $OCOCH_2CH(CH_2OH)CH_3$.

Table II.6 ^{1}H and ^{13}C assignments of 4'-hydroxy T-2 in $CDCL_3$

Atom	^{1}H	^{13}C
2	3.7	78.71
3	4.15	78.29
4	5.38, 5.35	84.46
5		48.32
6		42.89
7	2.37-2.45 1.85-1.94	
8	5.30, 5.29	68.26
10	5.81	123.83
11	4.38	67.23
12		64.28
13	3.05 2.79	47.18
14	0.807, 0.803	6.82, 6.74
15	4.29, 4.26 4.08, 4.06	
16	1.75	20.35
1'		172.96, 172.72
2'	2.1-2.2	38.89, 38.63
3'	1.6-1.8	33.19
4'	3.58 3.42	67.35
5'	0.96	16.72

Figure II.9 Positive CI mass spectrum of the TMS ether derivative of 4'-hydroxy T-2.

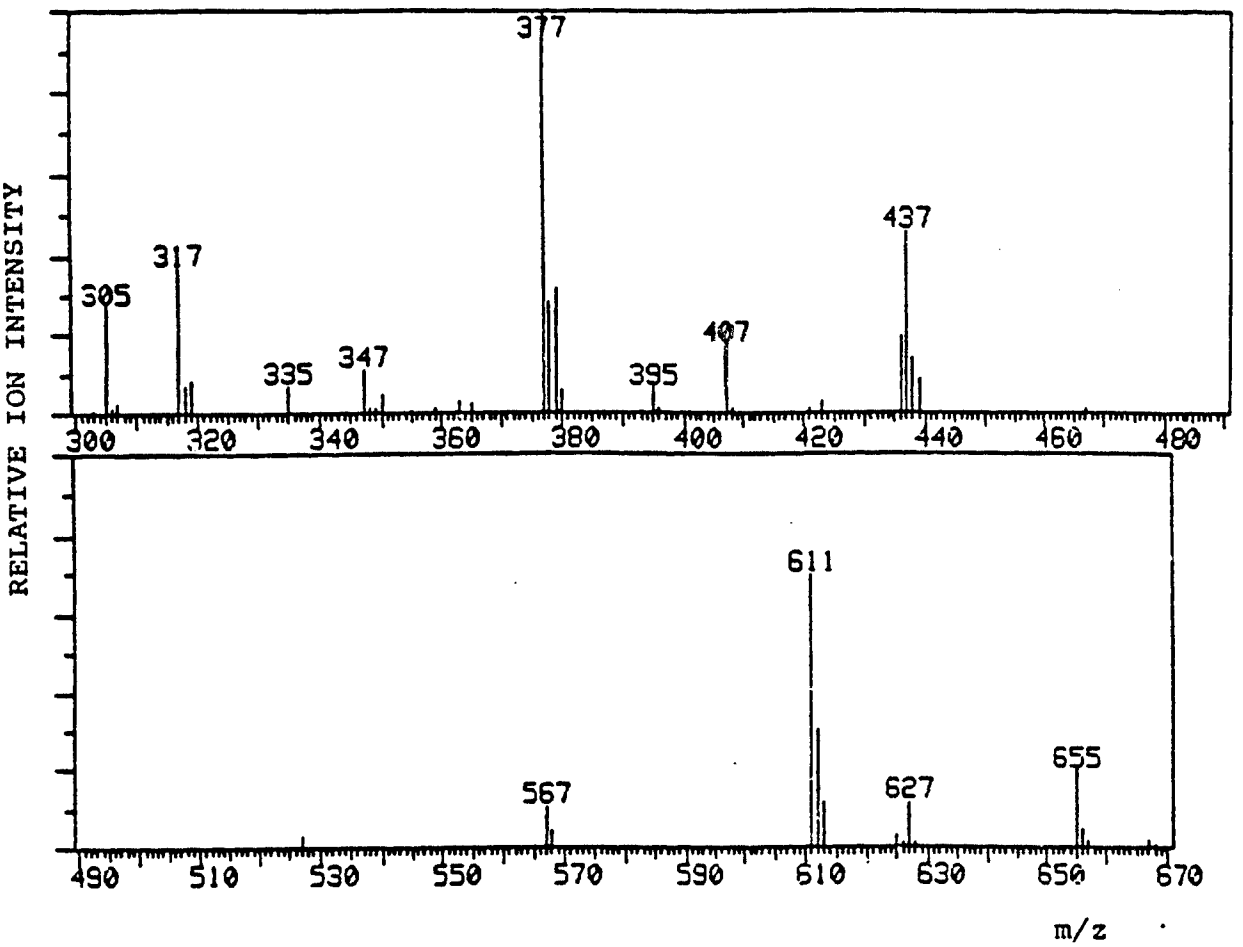


Figure II.10 A. Positive CI mass spectrum of the TFA derivative of 4'hydroxy T-2.
B. Positive CI mass spectrum of the TFA derivative of 3'-hydroxy T-2.

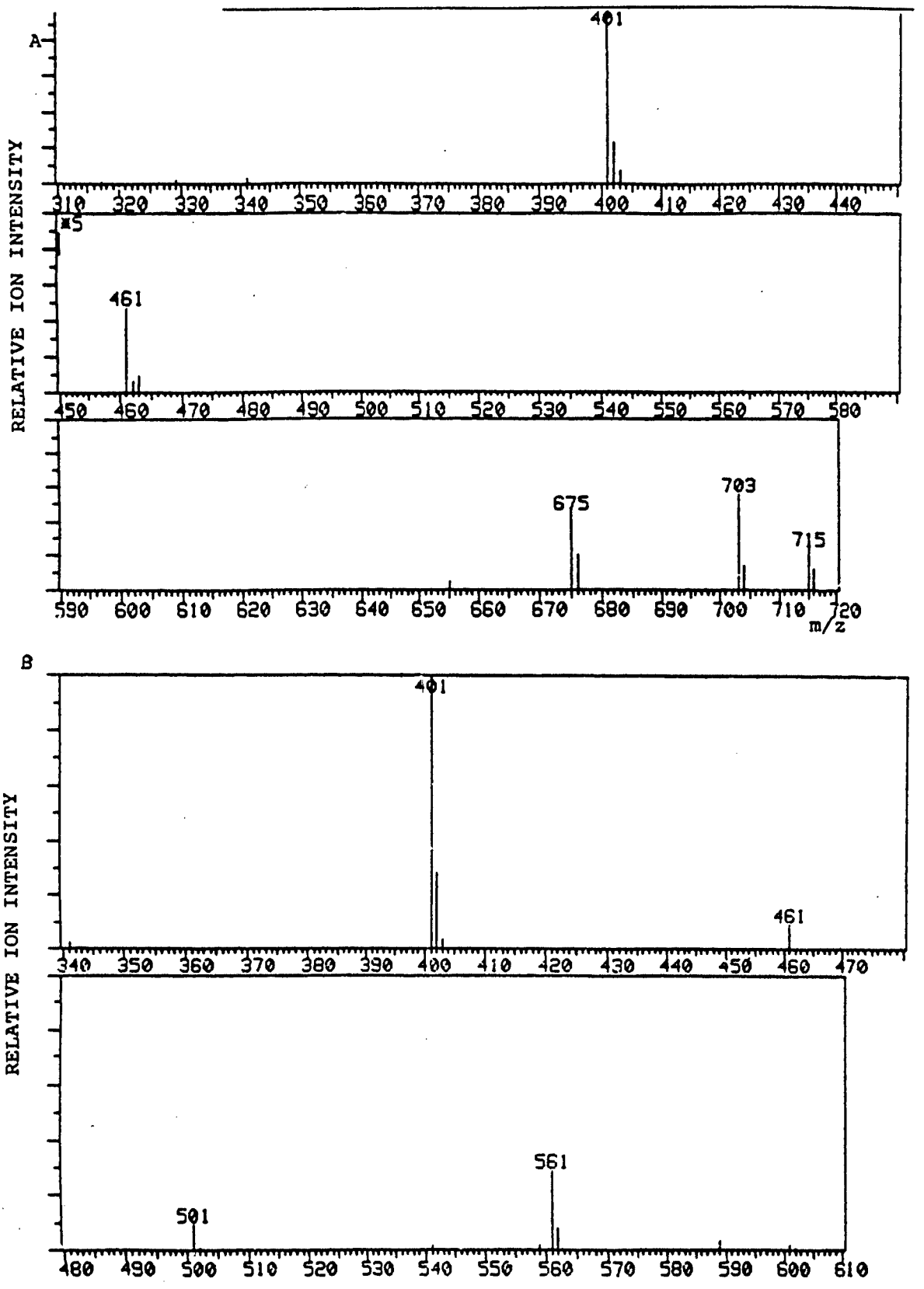


Figure II.11 Electron impact mass spectrum of 4'-hydroxy T-2.

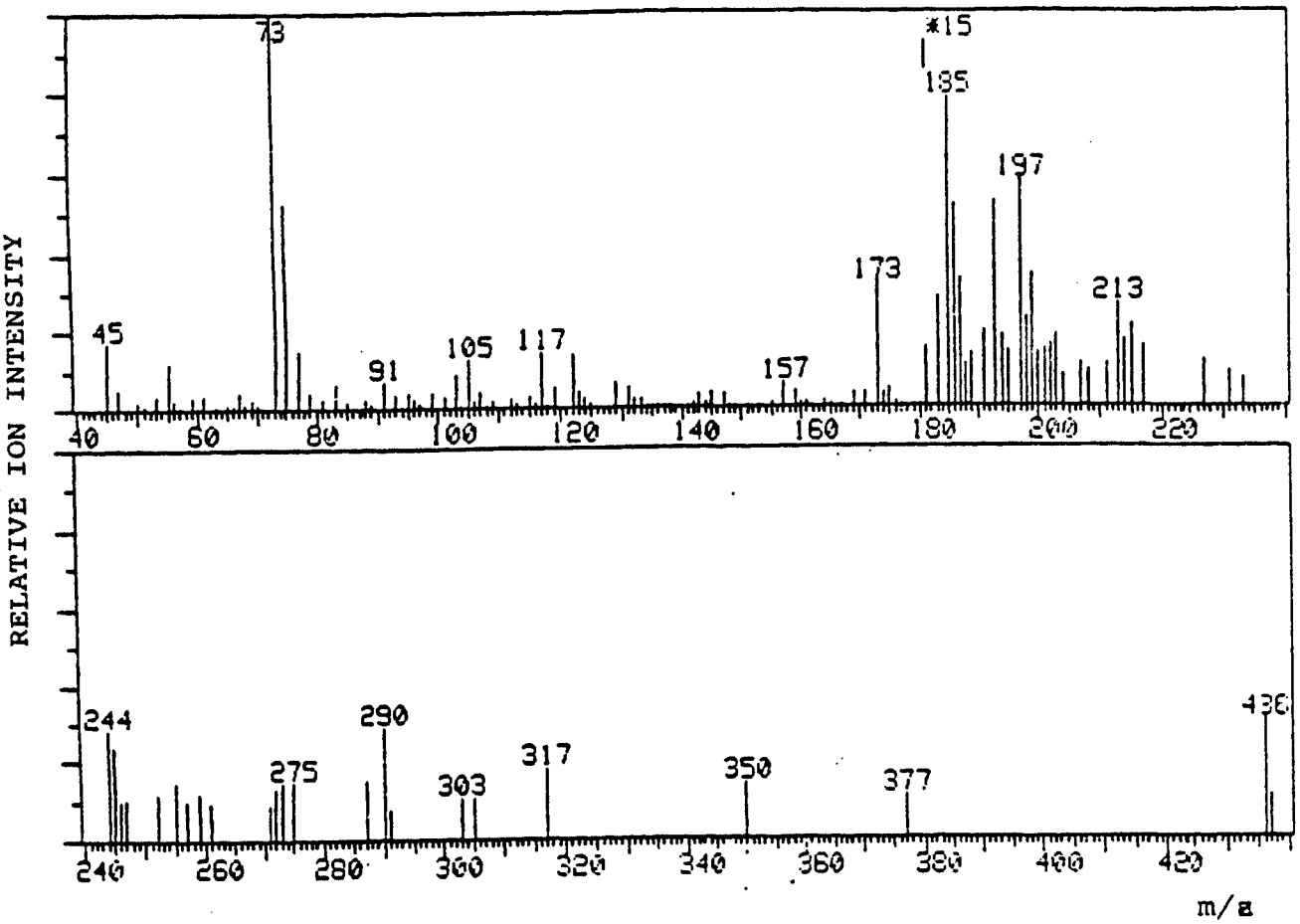
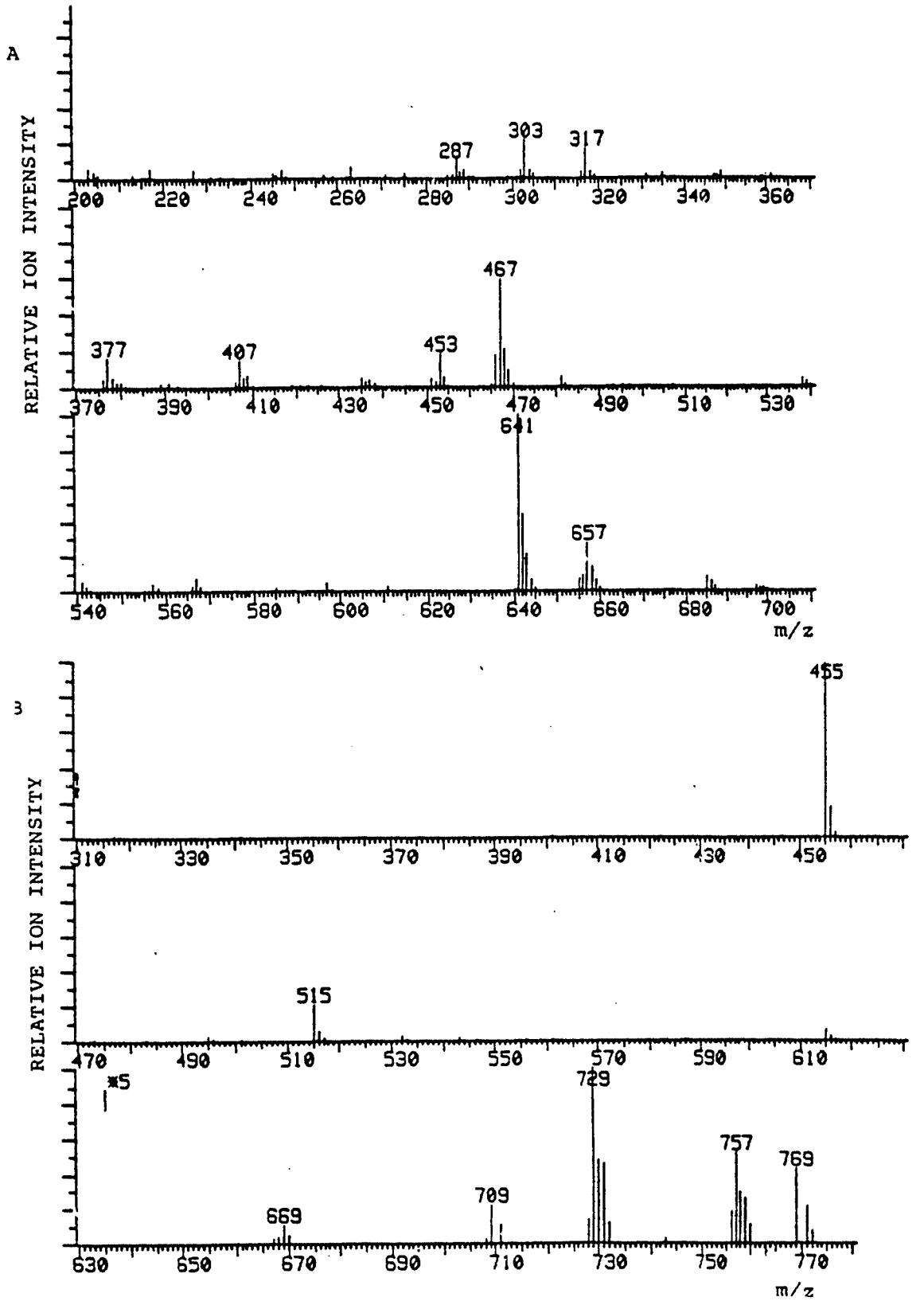


Figure II.12 A. Positive CI mass spectrum of the TMS derivative of MR-1.
B. Positive CI mass spectrum of the TFA derivative of MR-1.



2. In vivo

a Metabolism of T-2 toxin in rats: effects of dose, route, and time

by

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Abstract

Metabolic profiles of the excreta from rats following i.v., oral, and dermal administration of tritium-labeled T-2 toxin at 0.15 mg/kg and 0.60 mg/kg were determined. The major metabolites in urine were 3'OH HT-2, T-2 tetraol, and unknown metabolite M5, whereas the major metabolites in feces were deepoxy T-2 tetraol, 3'OH HT-2, and unknown metabolites M5, M7, and M9. The metabolite labeled M9 (major metabolite) was tentatively identified as deepoxy 3'OH HT-2. There was no significant effect on metabolic profiles due to dose, but there was a variable effect associated with the route of administration. The increase over time of appreciable levels of deepoxy metabolites as a percentage of extracted radioactivity was both consistent and statistically significant.

Introduction

The trichothecene mycotoxins are a chemical group of fungal metabolites characterized by a 12, 13-epoxytrichothec-9-ene skeleton. T-2 toxin, one of over 40 naturally occurring trichothecenes, is a toxic metabolite produced primarily by species of Fusarium (Bamburg and Strong, 1971). Serious mycotoxicoses, including moldy corn toxicosis in the U.S. and fusariotoxicosis in Canada, have been attributed to T-2 toxin (Hsu et al., 1972; Puls and Greenway, 1976). This toxin was also possibly involved in the bean-hull toxicosis of farm animals in Japan (Ueno et al., 1972). In addition, alimentary toxic aleukia, which has been a human health problem in Russia, was found to be associated primarily with the ingestion of moldy cereals infected with T-2 toxin producing strains of Fusarium (Joffe, 1971). The signs of trichothecene intoxication included emesis, decreased weight gain, lethargy, diarrhea, feed refusal, necrosis, lowered immunity, hemorrhage, and death (Hsu et al., 1972; Beasley, 1986; Kosuri et al., 1970; Osweiller et al., 1981; Boonchuvit, 1975; Wyatt et al., 1973; Glavits et al., 1983). Trichothecene mycotoxins, including T-2 toxin, and their effects on humans have received considerable international attention because of their alleged use in chemical warfare as the agent "Yellow Rain" in Southeast Asia (Watson et al., 1984).

The toxin, when administered to rodents, chickens, cattle, and swine, is rapidly metabolized into various products. T-2 toxin is rapidly metabolized and eliminated in a feces to urine ratio of 3:1 in mice, 5:1 in rats, and 1:4 in guinea pigs (Matsumoto et al., 1978; Pace et al., 1985).

The major metabolites isolated from urine and feces of a lactating cow after daily oral administration of T-2 toxin were 3'OH HT-2, 3'OH T-2 toxin, and 3'OH 7-OH HT-2 (Pawlosky and Mirocha, 1984; Yoshizawa et al., 1981; Yoshizawa et al., 1982). In a previous study using rats given an oral dose of T-2 toxin, the major metabolites isolated from feces were HT-2 and T-2 toxin at 2.7 and 7.5% of the administered dose, respectively, whereas 2 unknown metabolites which represented 25.8 and 9.1% of the dose were also present (Matsumoto et al., 1978). In urine from rats administered T-2 toxin, neosolaniol, HT-2, and three unknown metabolites which accounted for less than 8% of the administered dose were identified (Matsumoto et al., 1978). The metabolites of T-2 toxin in the excreta of chickens, following oral administration were, T-2 toxin, HT-2, neosolaniol, T-2 tetraol and several unknown metabolites. These were later identified as 3'OH T-2 toxin, 3'OH HT-2, 8 acetoxy, and 15 acetoxy T-2 tetraol (Visconti and Mirocha, 1985). In guinea pigs administered T-2 toxin i.m., the major urinary metabolites were identified as T-2 tetraol, 4-deacetylneosolaniol, and 3'OH HT-2. In bile, the major metabolites were HT-2, 4-deacetylneosolaniol, 3'OH T-2 triol, and 3'OH HT-2 (Pace et al., 1985). In swine administered T-2 toxin i.v., the major urinary metabolites were 3'OH HT-2, and T-2 triol plus the glucuronide conjugates of HT-2, 3'OH T-2, 3'OH HT-2, and T-2 toxin (Corley et al., 1985). Recently, deepoxy metabolites of 3'OH HT-2 including deepoxy T-2 tetraol, deepoxy 3'OH HT-2 and deepoxy 3'OH T-2 triol were identified in rat feces (Yoshizawa et al., 1985).

It is clear from the literature that the metabolism of T-2 toxin follows four distinct pathways: hydrolysis, hydroxylation, deepoxidation, and glucuronidation (Ohta et al., 1977; Yoshizawa et al., 1984; Yoshizawa et al., 1985; Corley et al., 1985). The purpose of this study was to determine the effects of dose, route of administration, and time on the first three metabolic pathways.

Experimental Section

Reference Standards

Tritium-labeled T-2 toxin (labeled in the C-3 position, radiopurity greater than 98%, and having a specific activity of 500 mCi/mmole) was obtained from Amersham Corporation, Arlington Heights, IL. Unlabeled standards of T-2 toxin, neosolaniol, HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol were produced from cultures of F. tricinctum in our laboratory.

Tritium-labeled T-2 toxin was used to prepare labeled standards of HT-2, T-2 triol, and T-2 tetraol by alkaline hydrolysis (Wei et al., 1971). Tritium-labeled 3'OH HT-2 and 3'OH T-2 triol were prepared by alkaline hydrolysis from 3'OH T-2 toxin which was prepared by incubation of labeled T-2 toxin with S-9 rat liver hemogenates (Knupp et al., 1985). Radiolabeled deepoxy derivatives of HT-2, T-2 triol and T-2 tetraol were prepared from tritium-labeled T-2 toxin using bovine rumen microflora (Swanson et al., 1987).

Animal Treatment

Male Sprague-Dawley rats weighing 200 to 250 g were obtained from Harlan Sprague-Dawley Inc., St. Louis, MO. Rats were individually

housed in metabolic cages (Nalge Company, Rochester, NY) and allowed to acclimate to a 12-hour day/night cycle for 7 days prior to dosing. Feed and water were provided ad libitum except for 12 hours predosing when it was removed. Six days following dosing all animals were killed by cervical dislocation.

Animal Dosing

Each rat was given 10 µCi tritium-labeled T-2 toxin at either 0.15 mg/kg or 0.60 mg/kg of body weight. Intravenous doses were dissolved in 0.25 ml of 50% ethanol/water and administered through a tail vein. The oral doses were dissolved in 0.25 ml of 50% ethanol/water and administered by gavage. Dermal doses were applied to an area approximately 1 cm^2 located between the scapulas in 0.1 ml of 90% DMSO/water. Each dose and route combination was repeated three times for a total of 18 rats.

Sample Handling

Feces and urine were collected every 6 hours and stored at -20°C until used for analysis.

Determination of Total Radioactivity

The total radioactivity in urine was determined by adding 0.1 ml plus 0.4 ml water directly to 5 ml of Aquasol-2® liquid scintillation cocktail (New England Nuclear Corp., Boston, MA). Quench correction was done by spiking another aliquot of urine with tritium-labeled T-2 toxin. The total radioactivity in feces was determined by first homogenizing 1.0 g in 20 ml of 0.1 M acetate buffer (pH 3.8). A 0.1 ml aliquot was removed and added to a 7 ml glass vial containing 0.1 ml perchloric acid (60%) and 0.2 ml hydrogen peroxide (30%). The vials were capped tightly and heated at 60°C for 24 hours. Following heating, the vials were allowed to cool, and 5 ml Aquasol-2® was added. Spiked feces samples were treated identically to allow for correction of the quenching effect from perchloric acid. Urine and feces samples were counted on a Packard Tri-Carb 300M Liquid Scintillation Counter (Packard Inst., Chicago, IL).

Urine Extraction

A 1.0 to 10.0 ml volume depending on the amount of total radioactivity present was diluted to 15 ml with water, followed by the addition of 5 g NaCl and 15 ml acetonitrile. Upon centrifugation at 2000 rpm, the top layer was removed. The extraction was repeated three times with acetonitrile and a fourth time with acetonitrile/acetone (1 + 1). The top layers were combined, and 100 ml of methylene chloride was added to drive residual water out of solution. Anhydrous sodium sulfate (approximately 50 g) was added to remove water (solutions appeared clear). Cupric carbonate (2.5 g) was added, and the samples were filtered through ash-free analytical filter pulp (Schleicher and Schuel, Inc., Keene, NH). The pulp and flasks were rinsed three times with 20 ml ethyl acetate. Samples were then concentrated to dryness and redissolved in 1 to 2 ml methanol, diluted with an equal volume of water, and filtered through a disposable membrane filter (Arco LC25, 0.2 micron; Gilman Sciences, Ann Arbor, MI) for HPLC radiochromatographic analysis.

Feces Extraction

To a 0.5 to 3.0 g sample was added 10 ml of 0.1 M acetate buffer (pH 3.8) and 10 ml acetonitrile. Each sample was mixed well and centrifuged at 2,000 rpm, and the aqueous acetonitrile layer was transferred to a 30 ml plastic centrifuge tube. The feces were extracted three times with 10 ml acetonitrile/water (1 + 1). To the combined aqueous acetonitrile portions was added 5 g NaCl followed by shaking and centrifugation to allow the phases to separate. The acetonitrile layer was removed, and the extraction was repeated three times with 10 ml acetonitrile and a fourth time with acetonitrile/ acetone (1 + 1). The acetonitrile extracts were diluted with methylene chloride and processed as described above for urine.

HPLC Radiochrmoatography

An HPLC system (Perkin Elmer Series 4, Norwalk, CT) was equipped with a 15 cm x 4.6 mm id column packed with 5 µ C18 (Econosphere, Alltech Assoc., Deerfield, IL). A 20 to 90% methanol in water linear gradient over 30 minutes at a flow rate of 1.0 ml/minute was used to separate metabolites. A sample volume at 100 µl containing approximately 100,000 cpm was injected onto the column. Fractions (0.2 ml) were collected and assayed for radioactivity in Scinti Verse LC® liquid scintillation cocktail (Fisher Scientific Co., Itasca, IL). Due to the efficient clean-up from HPLC, the effect of quench was not evident in the fractions assayed for radioactivity.

TLC Chromatography

Aliquots of samples were spotted into the outer channels of a precoated silica gel TLC plate (5 x 20 cm, 0.25 mm gel thickness, J. T. Baker) which was activated for 1 hour at 110°C. Plates were developed in chloroform/methanol (9 + 1) and allowed to air dry. To obtain radiochromatographic profiles, 1 to 2 mm bands were scraped from the TLC plate directly into scintillation vials. A 0.1 ml volume of water was added to each vial, followed by 0.25 ml methanol. Samples were then counted in 5 ml Aquasol-2®. Standards were visualized under long wave (365 nm) UV lamp after having been sprayed with 30% sulfuric acid in methanol and heated at 110°C for 35 minutes.

Statistical Analysis

Analysis of variance (ANOVA) using the percentage of extracted radioactivity (perofext) as the dependent variable was used. A value of $p < 0.05$ was used to indicate significance. Tukey's Studentized Range Test ($p < 0.05$) was used for comparisons between route, dose, and time.

Results and Discussion

Methodology

The analytical procedures developed and applied in this study were well suited for the analysis of ^{3}H T-2 toxin and metabolites which have a wide range of polarities as shown in Table II.7. Previously published methods for the analysis of T-2 toxin and metabolites in

excreta involved the use of Amberlite XAD-2 for the extraction of metabolites and Florisil and/or C-18 for the cleanup of samples prior to thin layer chromatography (Yoshizawa et al., 1980). In these studies, a solvent partition into acetonitrile followed by a cupric carbonate cleanup step were used. The use of cupric carbonate in this study provided a simple and rapid alternative to the use of Florisil and C-18. Significant cleanup of urine and feces extracts was also achieved by precipitating the oily residue after cupric carbonate cleanup with aqueous methanol, followed by filtration through 0.2 μ filters. The replacement of TLC with reverse phase HPLC greatly increased reproducibility and resolution.

Percent recoveries from spiked urine samples were 46.0 ± 1.2, 74.6 ± 2.0, 73.4 ± 1.3, and 75.1 ± 2.0 ($\bar{x}$ ± SE, n = 4) for T-2 tetraol, T-2 triol, HT-2, and T-2 toxin, respectively. Percent recoveries from spiked fecal samples were 42.6 ± 1.7, 71.2 ± 2.1, 70.6 ± 1.9, and 73.1 ± 2.3 ($\bar{x}$ ± SE, n = 4) for T-2 tetraol, T-2 triol, HT-2, and T-2 toxin, respectively. The reproducibility of the extraction procedure for overall radioactivity was tested using a representative urine sample from a treated animal. The percent recovery of radioactivity was 59.0 ± 2.1 ($\bar{x}$ ± SE, n = 12). Considering the wide range of polarity between T-2 toxin and its metabolites, this standard error (SE) was quite low.

Excretion of Total Radioactivity

The total cumulative excretion of radioactivity in rat urine and feces is shown in Figure II.13. The excretion of radioactivity was rapid and nearly complete (greater than 95%) 72 hours after administration of labeled T-2 toxin in orally dosed rats. This rapid excretion of T-2 toxin and metabolites was consistent with that reported in other species (Matsumoto et al., 1978; Pace et al., 1985; Yoshizawa et al., 1980; Yoshizawa et al., 1981).

In rats given an i.v. injection of T-2 toxin at 0.15 mg/kg, the excretion of radioactivity was very rapid and nearly complete after 72 hours, but in rats given 0.6 mg/kg, the excretion was less than 80% after 72 hours. Extensive vascular damage was noted in rats given T-2 toxin at this concentration which possibly resulted in decreased absorption of T-2 toxin.

In dermally-dosed rats, less than 60% of the radioactivity was excreted 72 hours after administration. Excretion studies using swine as a model have shown that the skin and surrounding fat act as a depository for T-2 toxin (Pang et al., 1987). This may account for the decreased excretion of radioactivity in dermally dosed rats. As in i.v. dosed rats, the excretion of radioactivity was less in the high dose (0.6 mg/kg) rats than the low dose rats.

Metabolite Determination

HPLC analysis of urine and fecal extracts resulted in 16 different radioactive peaks. A total of 68.3% of the extracted radioactivity in urine averaged over dose, route, and time had HPLC retention times identical to standards of T-2 toxin (5.6%), HT-2 (8.9%), 3'OH HT-2 (29.3%), 3'OH T-2 toxin (3.2%), and T-2 tetraol (21.3%). Two unknown metabolites labeled M5 (7.5%) and M9 (13.8%) accounted for an

additional 21.3% of the extracted radioactivity. Metabolite M9 was further characterized by its hydrolysis to deepoxy tetraol and its co-migration using TLC with 3'OH HT-2. Based on retention time data, it was postulated that M7 was deepoxy 3'OH T-2 triol, that M5 was 3'OH T-2 triol, and M9 was deepoxy 3'OH HT-2.

In feces, a total of 29.1% of the radioactivity averaged over dose, route, and time had HPLC retention times identical to deepoxy HT-2 (3.4%), 3'OH HT-2 (15.1%), and deepoxy T-2 tetraol (10.6%). Three unknown metabolites accounted for an additional 61.4% of the extracted radioactivity. These metabolites were labeled M5 (5.2%), M7 (9.7%), and M9 (46.5%).

Chromatographic analysis of the radioactivity in the excreta of rats revealed that the major metabolites regardless of dose or route were 3'OH HT-2, HT-2, T-2 tetraol, deepoxy T-2 tetraol, and several unknowns. The hydrolysis of T-2 toxin to T-2 tetraol via several intermediates, as well as the hydroxylation of T-2 toxin and HT-2 at the 3' position, have been previously reported (Yoshizawa et al., 1980; Visconti and Mirocha, 1985; Yoshizawa et al., 1984). The deepoxidation pathway in vivo has been reported using 3'OH HT-2 and in vitro using T-2 toxin (Yoshizawa et al., 1985,1985a). This study presents evidence indicating that deepoxidation is an important in vivo metabolic pathway for T-2 toxin in rats.

Deepoxidation results in significant detoxification of T-2 toxin. The deepoxy derivatives of HT-2, T-2 tetraol, and T-2 triol exhibited no toxicity (LD_{50} greater than 5000 mg/ml) to brine shrimp (Swanson et al., 1987).

The metabolic profiles (HPLC radiochromatograms) for representative urine and fecal extracts are shown in Figure II.14. The metabolic profiles expressed as a percent of the extracted radioactivity for route and dose over time are given in Tables 2 and 3.

The extracted radioactivity represented 52.4 $\pm$ 2.4 and 43.9 $\pm$ 1.4 ($\bar{x}$ $\pm$ SE, n = 58) percent of the total radioactivity in urine and feces, respectively. Although no differences were noted in the extracted radioactivity between doses and routes, a decrease in extracted radioactivity was observed over time. This decreasing trend was due possibly to an increased amount of nonextracted polar metabolites, such as glucuronide conjugates, and/or binding. Although conjugated metabolites were not investigated in this study, they have been reported in swine (Corley et al., 1985).

Statistical Interpretation for Urine

There were no significant differences in metabolic profiles expressed as a percent of extracted radioactivity due to dose. However, route and time and their interactions were significant for several metabolites as shown in Figures 3 to 5. The percentage of HT-2 averaged over dose and time in orally dosed rats was significantly lower than in dermally and i.v. dosed rats. This effect was also significant but reversed for metabolite M9. The percentage of metabolite M9 averaged over dose and time in orally and i.v. dosed rats was significantly higher than that for dermally dosed rats.

For T-2 tetraol, the percentage averaged over dose and time was greatest for i.v. dosed rats, but for 3'OH T-2 toxin, the percentage was less for i.v. dosed rats than orally or dermally dosed rats (see Figure II.15).

A significant difference in the percentage of extracted radioactivity over time was noted as shown in Figure II.16. For HT-2, the percentage averaged over route and dose for Day 1 was greater than for Day 3. For M9, the percentages for Days 2 and 3 were greater than those for Day 1.

Although no main effects were seen for T-2 toxin, the interaction between route and day was significant. The percentage of T-2 toxin in the urine of orally and i.v. dosed rats was significantly less than in dermally dosed rats as shown in Figure II.17.

Statistical Interpretation for Feces

Animals given the high dose had significantly greater fractions of the dose metabolized to deepoxy T-2 tetraol and 3'OH HT-2 than animals given the low dose. However, the interactions between route, time, and dose were complex for these two metabolites. The statistical results for feces are summarized in Figures II.18 to II.20. Examination of the percentage of extracted radioactivity over dose and time indicated that the percentage of deepoxy T-2 tetraol was significantly higher in feces from i.v. dosed rats than orally or dermally dosed rats; but for 3'OH HT-2, the percentage from orally dosed rats was higher than from i.v. and dermally dosed rats. For HT-2 and deepoxy HT-2, the percentages averaged over dose and time were significantly higher in dermally dosed rats than i.v. and orally dosed rats (Figure II.19).

The effect of time on the metabolic profiles was the most significant effect detected in feces. For 3'OH HT-2 and HT-2, the percentages averaged over dose and route for Day 1 were higher than for Day 2; but for deepoxy T-2 tetraol, Days 2 or 3 were significantly higher than Day 1 (see Figure II.20).

The application of HPLC as evidenced by this study was an excellent analytical tool for the isolation, separation, and identification of trichothecene metabolites. The capabilities of HPLC far outweigh those procedures such as TLC that have been used in previous studies.

Evidence from this study suggests that several significant interactions of route and time are important, whereas the effect of dose was not. The major metabolic pathways, hydrolysis, hydroxylation, and deepoxidation were not effected by the two dose levels used. The effect of route was significant for several metabolites. The higher levels of T-2 tetraol and deepoxy T-2 tetraol in i.v. dosed rats indicates that hydrolysis and deepoxidation serve as the major metabolic pathways by this route. The lower value of 3'OH HT-2 in i.v. dosed rats supports this conclusion. The increase of T-2 toxin over time as a percent of extracted radioactivity in urine from rats administered T-2 toxin dermally is not fully understood, but may arise from the slow release of toxin from the site of application. In swine topically administered T-2 toxin, the skin and fat acted as a reservoir for the toxin; residues of the parent toxin and the

acylation moduclus were detected 14 days at the application site 14 days after exposure (Pang et al., 1987).

The most significant effect was that of time. The percentage of extracted radioactivity associated with deepoxy metabolites increased significantly over time, regardless of the route or dose. Although the epoxy ring in a trichothecene nucleus seems to be an important moiety for the toxic action of T-2 toxin, the toxicological estimation of deepoxytrichothecenes remains to be established. Deepoxidation has been reported to be a detoxification pathway and in vitro studies suggest that intestinal microflora are responsible (Yoshizawa et al., 1985). Alterations of intestinal microflora may result in an increased toxicity of animals to T-2 toxin.

Acknowledgement

This project was supported in part by the U.S. Army Medical Research and Development Command, Contract No. DAMD 17-85-C-5224.

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Table II.7 Chemical structures of T-2 toxin and selected metabolites.

Name	R^1	R^2	R^3	R^4	R^a
T-2 toxin	OH	OAc	OAc	$OCOCH_2CH(CH_3)_3$	-O-
3'OH T-2	OH	OAc	OAc	$OCOCH_2C(OH)(CH_3)_3$	-O-
Neosolaniol	OH	OAc	OAc	OH	-O-
HT-2	OH	OH	OAc	$OCOCH_2CH(CH_3)_3$	-O-
3'OH HT-2	OH	OH	OAc	$OCOCH_2COH(CH_3)_3$	-O-
T-2 triol	OH	OH	OH	$OCOCH_2CH(CH_3)_3$	-O-
T-2 tetraol	OH	OH	OH	OH	-O-
de 3'OH HT-2	OH	OH	OAc	$OCOCH_2C(OH)(CH_3)_3$	$=CH_2$
de T-2 tetraol	OH	OH	OH	OH	$=CH_2$

[a]Represents the epoxide group at the 12, 13 position or its replacement following deepoxidation.

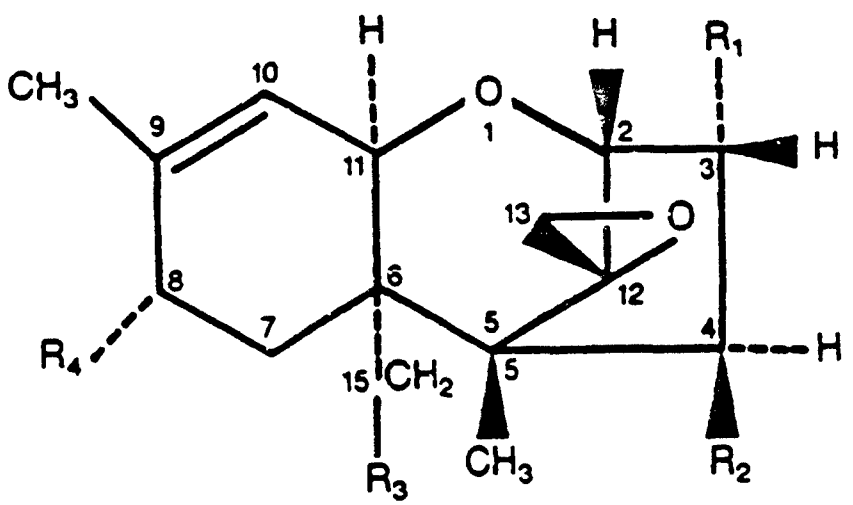

Table II.8 Metabolic profiles expressed as percent of extracted radioactivity in urine from rats administered tritium-labeled T-2 toxin.

		ROUTE OF ADMINISTRATION[1]					
		Oral		I.V.		Dermal	
Metabolite	Day	0.15 mg/kg	0.6 mg/kg	0.15 mg/kg	0.6 mg/kg	0.15 mg/kg	0.6 mg/kg
HT-2	1	3.9 ± 0.1	6.1 ± 1.0	19.8 ± 0.4	11.7 ± 1.8	13.2 ± 1.7	18.6 ± 0.5
	2	4.4 ± 1.8	9.1 ± 4.2	8.8 ± 1.0	6.3 ± 0.7	8.0 ± 1.1	12.1 ± 0.2
	3	4.2 ± 1.4	3.7 ± 1.9	7.6 ± 1.7	7.3 ± 1.7	6.3 ± 0.1	8.6 ± 1.1
3'OH T-2	1	4.6 ± 1.3	3.3 ± 1.0	2.7 ± 0.6	3.2 ± 0.3	4.6 ± 0.2	6.2 ± 1.0
	2	5.5 ± 0.6	0.3 ± 0.3	1.1 ± 1.1	1.8 ± 0.9	4.2 ± 0.8	3.7 ± 0.9
	3	3.5 ± 2.8	2.1 ± 1.0	3.0 ± 0.6	0.8 ± 0.8	4.0 ± 0.8	3.5 ± 0.4
M9	1	7.5 ± 1.0	5.6 ± 1.1	6.9 ± 0.7	5.1 ± 1.4	6.4 ± 0.8	16.9 ± 1.2
	2	26.5 ± 1.3	21.2 ± 3.9	27.3 ± 2.6	30.3 ± 3.4	9.4 ± 0.3	12.7 ± 1.5
	3	16.4 ± 4.0	19.4 ± 8.0	13.7 ± 3.8	17.2 ± 5.8	3.8 ± 0.3	10.7 ± 1.8
3'OH HT-2	1	32.1 ± 3.6	42.4 ± 2.6	24.6 ± 1.2	22.6 ± 4.4	30.3 ± 1.4	26.2 ± 2.9
	2	27.0 ± 2.2	33.9 ± 7.3	23.2 ± 0.7	22.4 ± 1.2	36.3 ± 1.8	34.1 ± 2.5
	3	28.7 ± 4.2	36.7 ± 3.6	22.5 ± 5.3	25.4 ± 6.7	29.2 ± 3.4	30.1 ± 1.6
M5	1	12.8 ± 2.8	14.6 ± 1.5	6.9 ± 1.3	12.3 ± 3.7	11.6 ± 1.6	7.1 ± 0.7
	2	5.0 ± 2.3	5.2 ± 2.5	2.4 ± 0.7	4.3 ± 1.2	8.3 ± 1.8	4.7 ± 1.4
	3	7.0 ± 0.6	10.1 ± 1.9	3.9 ± 2.0	7.2 ± 2.7	5.4 ± 1.4	6.1 ± 1.1
T-2 Tetraol	1	18.0 ± 4.7	20.3 ± 3.1	29.0 ± 1.6	29.2 ± 3.9	21.7 ± 1.2	22.6 ± 1.6
	2	19.4 ± 1.3	18.0 ± 3.2	28.7 ± 3.1	24.8 ± 3.6	15.0 ± 1.8	16.3 ± 0.6
	3	24.7 ± 0.9	13.0 ± 3.7	31.6 ± 4.0	23.0 ± 8.9	14.4 ± 2.9	17.0 ± 1.0

[1]Values reported as the mean ± SE (n = 3).

Table II.9 Metabolic profiles expressed as percent of extracted radioactivity in feces from rats administered tritium-labeled T-2 toxin.

		ROUTE OF ADMINISTRATION[1]					
		Oral		I.V.		Dermal	
Metabolite	Day	0.15 mg/kg	0.6 mg/kg	0.15 mg/kg	0.6 mg/kg	0.15 mg/kg	0.6 mg/kg
De HT-2	1	3.0 ± 1.0	1.6 ± 0.8	5.1 ± 1.7	2.8 ± 1.6	6.8 ± 1.6	5.9 ± 0.8
	2	2.3 ± 1.0	1.7 ± 0.5	2.3 ± 0.8	1.7 ± 1.2	3.8 ± 1.4	4.3 ± 0.4
	3	3.0 ± 1.0	0.6 ± 0.1	1.8 ± 1.1	1.7 ± 0.6	6.8 ± 1.6	5.0 ± 0.8
M9	1	49.7 ± 3.8	14.5 ± 2.4	40.3 ± 3.7	44.2 ± 2.8	43.0 ± 5.8	40.9 ± 7.3
	2	53.6 ± 3.5	60.1 ± 5.1	50.9 ± 2.3	45.3 ± 1.3	51.2 ± 4.4	56.2 ± 2.8
	3	46.6 ± 2.7	50.5 ± 6.8	42.1 ± 1.4	42.7 ± 3.3	48.5 ± 3.8	55.9 ± 1.2
3'OH HT-2	1	14.2 ± 1.0	50.2 ± 4.2	15.6 ± 5.0	7.7 ± 0.8	15.2 ± 5.1	19.1 ± 7.6
	2	8.9 ± 1.6	12.4 ± 3.3	12.8 ± 1.4	9.8 ± 1.3	8.5 ± 1.5	8.8 ± 1.8
	3	11.6 ± 0.2	18.9 ± 5.6	18.0 ± 2.7	14.0 ± 0.5	15.2 ± 2.9	10.1 ± 1.9
M7	1	9.7 ± 0.5	5.0 ± 3.0	9.4 ± 2.1	18.1 ± 3.1	6.2 ± 1.0	7.6 ± 2.6
	2	12.3 ± 0.7	8.3 ± 1.5	10.4 ± 1.9	13.3 ± 2.6	10.3 ± 1.4	11.0 ± 1.5
	3	10.0 ± 1.4	8.3 ± 2.5	9.2 ± 1.1	8.7 ± 3.8	6.4 ± 2.0	9.6 ± 1.3
M5	1	7.5 ± 1.1	9.1 ± 3.6	9.7 ± 2.0	4.3 ± 1.1	8.7 ± 2.4	6.8 ± 2.4
	2	4.4 ± 1.3	3.0 ± 1.3	4.7 ± 0.6	3.1 ± 1.0	3.7 ± 0.9	2.3 ± 0.8
	3	5.1 ± 1.7	4.2 ± 2.0	4.1 ± 0.5	3.7 ± 0.8	1.8 ± 0.1	1.5 ± 0.2
De Tol	1	6.7 ± 1.8	4.4 ± 1.4	9.1 ± 1.9	2.3 ± 0.3	2.2 ± 1.0	2.3 ± 0.5
	2	8.4 ± 1.0	9.8 ± 1.7	11.9 ± 0.5	20.3 ± 1.7	9.8 ± 0.5	10.3 ± 1.2
	3	10.4 ± 0.4	9.7 ± 0.8	12.6 ± 0.9	20.9 ± 0.1	8.0 ± 0.6	8.7 ± 0.3

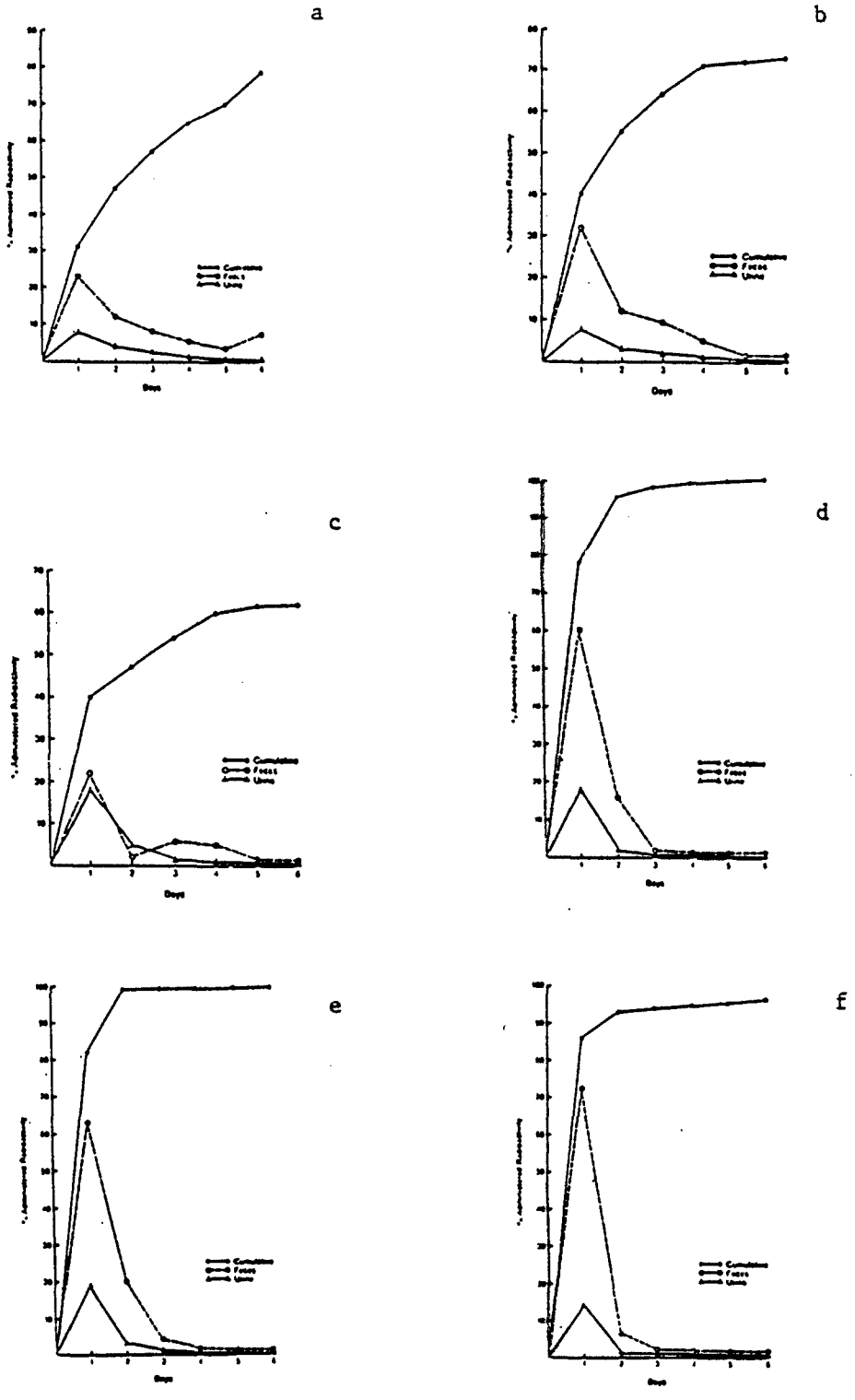

Figure II.13 Excretion of radioactivity in urine and feces from rats administered tritium-labeled T-2 toxin at 0.6 mg/kg dermally (a), i.v. (c), and orally (e) or 0.15 mg/kg dermally (b), i.v. (d), and orally (f).

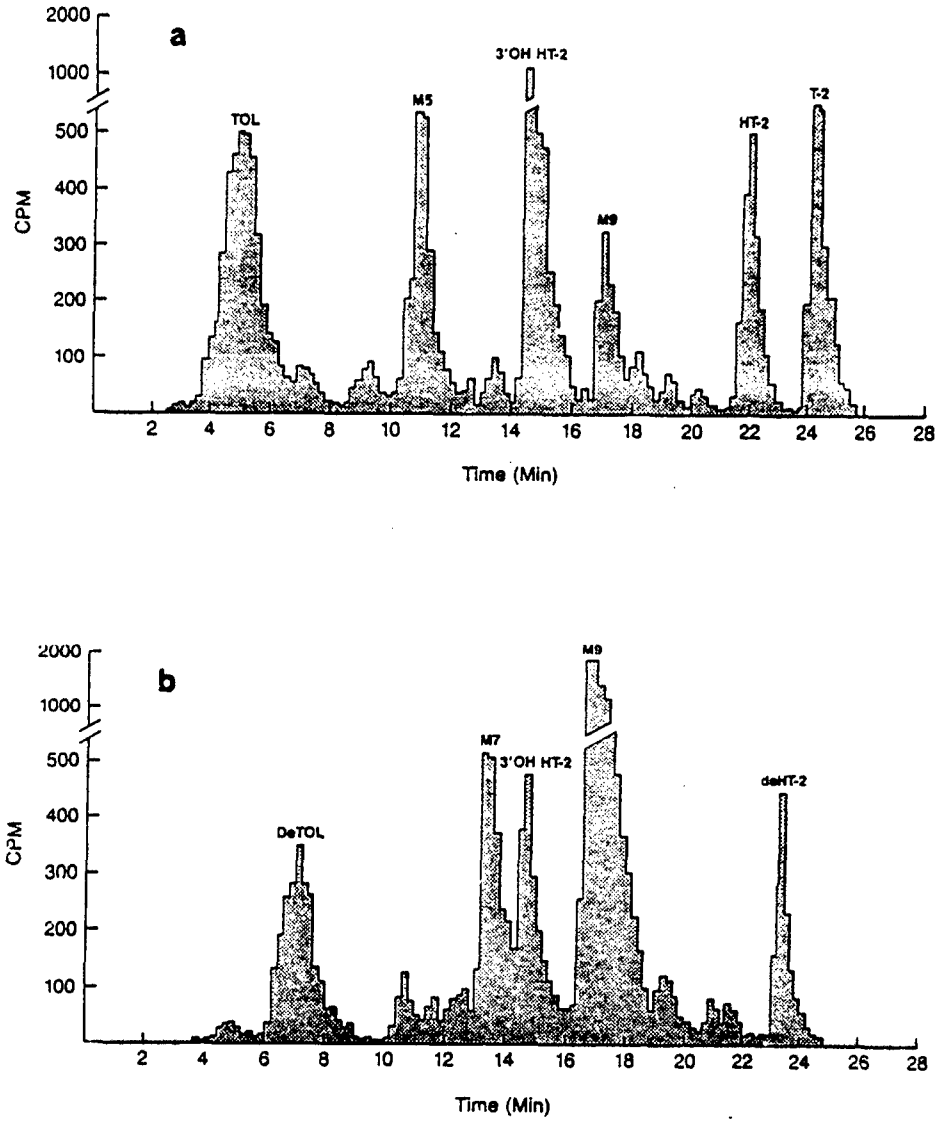

Figure II.14 Representative HPLC chromatograms of urine (a) and feces (b) from rats administered tritium-labeled T-2 toxin.

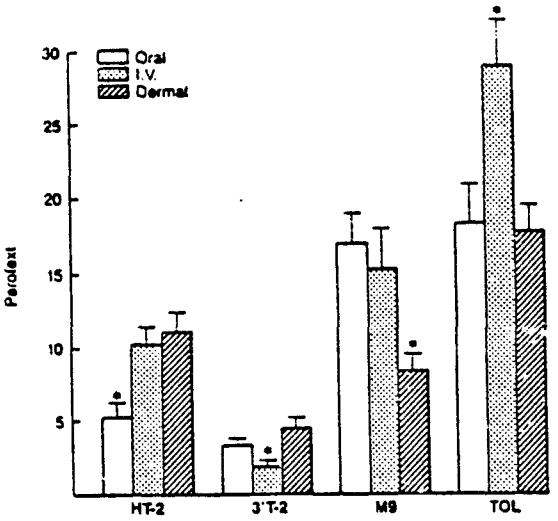

Figure II.15 Effect of route on selected metabolites in rat urine (* indicates $p < 0.05$) expressed as percent of extracted radioactivity (Perdext)

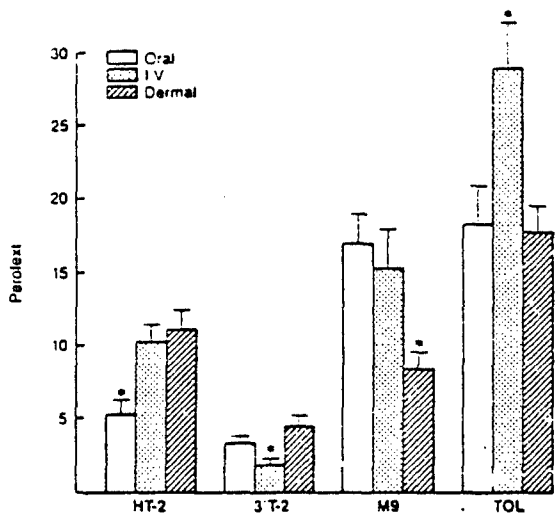

Figure II.15 Effect of route on selected metabolites in rat urine (* indicates $p < 0.05$) expressed as percent of extracted radioactivity (Perofext).

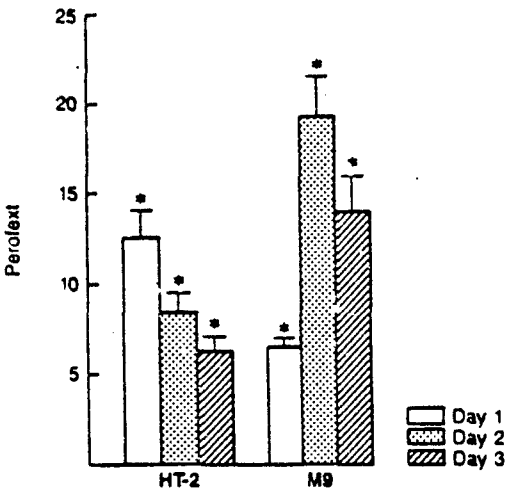

Figure II.16 Effect of time on selected metabolites in rat urine (* indicates $p < 0.05$) expressed as percent of extracted radioactivity (Perofext).

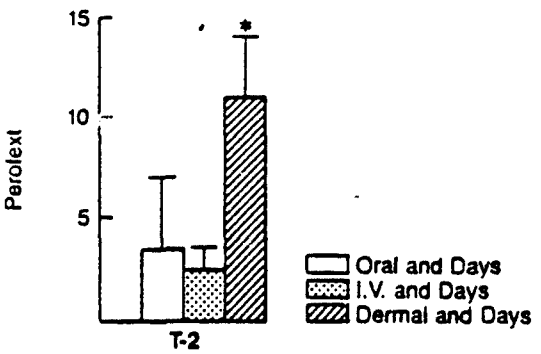

Figure II.17 Effect of route and time averaged over dose (* indicates $p < 0.05$) for T-2 toxin in rat urine expressed as percent of extracted radicactivity (Perofext).

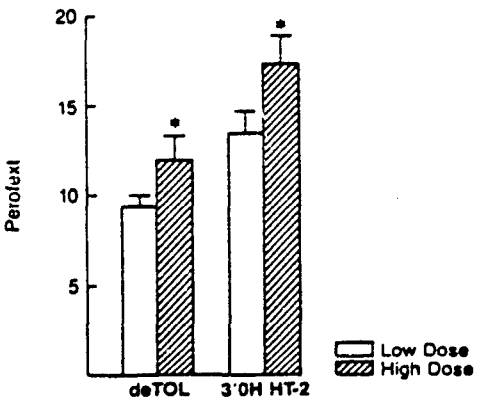

Figure II.18 Effect of dose on selected metabolites in rat feces (* indicates $p < 0.05$) expressed as percent of extracted radioactivity (Perofext).

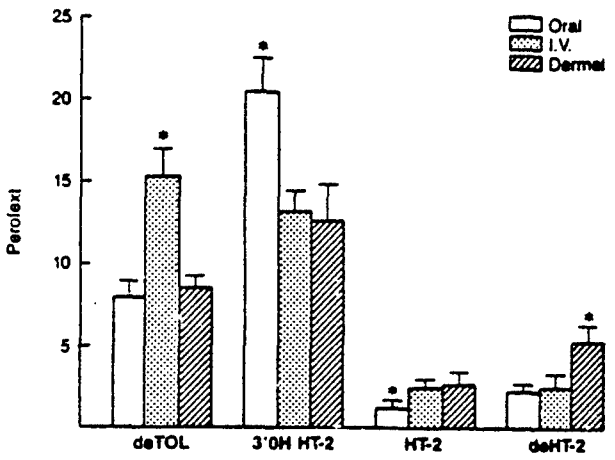

Figure II.19 Effect of route on selected metabolites in rat feces (* indicates p < 0.05) expressed as percent of extracted radioactivity (Perofext).

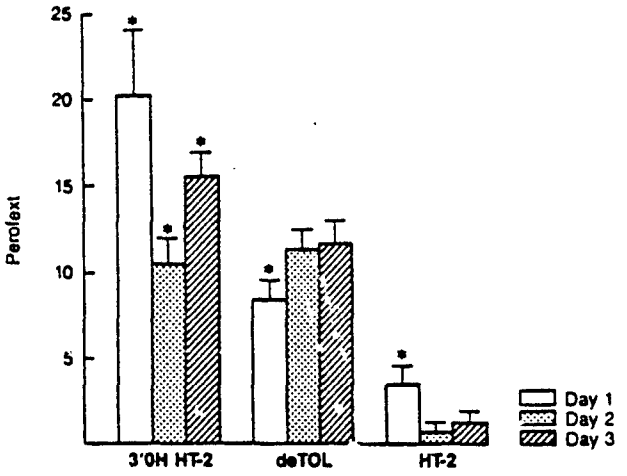

Figure II.20 Effect of time on selected metabolites in rat feces (* indicates $p < 0.05$) expressed as percent of extracted radioactivity (Perofext).

b. Effect of antibiotic therapy on the toxicity of orally administered T-2 toxin in mice

Introduction

Reduction of the epoxide group in trichothecenes, also called deepoxidation, is one of the major routes of biotransformation in many different animal species. In susceptible species, deepoxidation reactions occur with all trichothecenes investigated to date, including diacetoxyscirpenol (Sakamoto et al., 1986), deoxynivalenol (Côté et al., 1986; Yoshizawa et al., 1983; Lake et al., 1987), and T-2 toxin (Chatterjee et al., 1986a,b; Pfeiffer, Swanson, and Buck, submitted for publication).

Deepoxidation is an effective single step detoxification reaction for trichothecene mycotoxins (Swanson et al., 1987b). Once the epoxide group has been reduced to a carbon-carbon double bond, both oral and dermal toxicity are essentially abolished. Epoxide reduction occurs through the action of both rumen and gastrointestinal anaerobic microorganisms (King et al., 1984; Swanson et al., 1987a,1988; Chatterjee et al., 1986a,b). Although the deepoxy trichothecenes have been demonstrated to be major excretion products in a variety of animal species and deepoxidation of trichothecenes results in a loss of toxicity, the role deepoxidation plays in the overall toxicity of animals exposed to trichothecenes has not been established.

Since deepoxidation of trichothecenes are the result of bacterial metabolism in the gut, antibiotic therapy should minimize or eliminated this reaction by severely reducing the bacterial population in the gastrointestinal tract. In the study described, we report the effects combined oral antibiotic therapy has on the acute toxicity of T-2 toxin orally administered to mice.

Experimental

Eighty-four male NIH Swiss mice weighing 24 to 30 g were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Animals were housed in groups of six and were acclimatized in cages for 1 week prior to initiation of experiments. One week prior to dosing with T-2 toxin the animals were divided into two treatment groups. Group 1 recieved water only and group 2 water amended with a mixture of antibiotics (4 mg/ml bacitracin, 4 mg/ml neomycin sulfate, and 0.3 mg/ml penicillin G). The antibiotic solutions were prepared fresh every 48 hours and treatment was continued for 1 week after dosing. Seven days after initiation of antibiotic therapy, both treatment groups were administered T-2 toxin (dissolved in 10% ethanol) by oral gavage at dosages of 0, 6, 8, 10, 12, 14, and 16 mg/kg body weight. Each dosage was administered to six mice. Deaths were recorded daily for 7 days. LD_{50} determinations were performed using the moving average method of Weil.

Results

No deaths were observed in any of the vehicle control groups. The mean LD_{50}s (standard deviation) for the T-2 toxin plus antibiotics and the T-2 toxin only groups were 12.53 (0.49) and 9.42 (0.29) mg/kg body weight, respectively. This difference was significant at

$P < 0.05$. See Table II.10 for lethality at each dosage and times of death.

Discussion

Treatment with combined oral antibiotics prior to exposure to T-2 toxin significantly reduced lethality induced by this mycotoxin. However, the mechanism of this protective effect has not been established. Alterations in biotransformation as a result of antibiotic therapy may be at least partially responsible for the observed effect. One of the most pronounced effects of antibiotic therapy, in general, is the reduction of overall microflora present in the gastrointestinal tract and alterations in the species of bacteria populating the intestines. Alterations in intestinal bacteria would most likely affect two major biotransformation pathways, deepoxidation and glucuronide hydrolysis.

Deepoxidation is a detoxification reaction accomplished via the action of anaerobic intestinal microflora in a number of animal species. Antibiotic therapy should result in decreased populations of intestinal microflora and an overall reduction in the level of this detoxification reaction. As a result one would expect to see an increase in toxicity if deepoxidation (a detoxification reaction) were the predominante biotransformation pathway involved in toxicity of T-2 toxin. This was not observed in the present study.

Metabolism of the trichothecene mycotoxins, and especially T-2 toxin, is very complex. To date at least 26 metabolites of T-2 toxin have been identified by a variety of researchers. Glucuronide conjugation has been demonstrated to be a major pathway of metabolism, not only for T-2 toxin (Gareiss et al., 1986; Corley et al., 1985,1986) but other trichothecenes as well, including diacetoxyscirpenol (Gareiss et al., 1986) and deoxynivalenol (Côté et al., 1986; Prelusky et al., 1986,1987; Lake et al., 1987). Most animal species investigated biotransform trichothecenes, at least a limited extent, via glucuronide conjugation.

It is well documented that intestinal microflora produce beta-glucuronidase within the gut and that this enzyme is responsible for the hydrolysis of many different xenobiotic conjugates excreted into the intestines via the bile. Hydrolysis of xenobiotic glucuronide conjugates results in the liberation of the free aglycone. If the aglycone is sufficiently lipophillic, reabsorption may take place resulting in enterohepatic recirculation. In instances where the xenobiotic in question is a toxic compound, disruption of the enterohepatic cycle can reduce toxicity by increasing the rate of elimination. T-2 toxin is excreted in the bile to a significant extent as the glucuronide conjugate and as glucuronide conjugates of related toxic metabolites such as HT-2 and 3'OH T-2 (Corley et al., 1985,1986; Gareiss et al., 1986; Pace et al., 1986). T-2 toxin and metabolites have been demonstrated to undergo enterohepatic recirculation in the rat (Coddington et al., unpublished data) and probably in other species such as swine and guinea pigs as well (Pace et al., 1986; Corley et al., 1986). Reduction in the enterohepatic recirculation of toxic metabolites and consequently enhanced rates of elimination may be one mechanism by which antibiotic therapy reduces toxicity of orally administered T-2

toxin. Additional studies comparing metabolite excretion profiles in animals exposed to T-2 toxin with and without antibiotic therapy are needed in order to determine whether the above hypothesis holds true.

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Table II.10a Lethality of mice administered T-2 toxin and treated with combined oral antibiotics

Dosage[a]	Number of dead animals per group Time after dosing (days) 1	2	3	4	5	6	7	Total[b]	Cummulative Total[c]
0	0	0	0	0	0	0	0	0/6	0
6	0	0	0	0	0	0	0	0/6	0
8	0	0	0	0	0	0	0	C/6	0
10	0	0	0	0	0	0	0	0/6	0
12	0	1	1	0	0	0	0	2/6	2
14	0	2	2	2	0	0	0	6/6	8
16	0	0	1	1	1	0	1	4/6	12

[a]T-2 toxin dosage in mg/kg body weight.
[b]Total nuber of animals dead
[c]Cummulative number of dead animals.

Table II.10b Lethality to mice administered T-2 toxin only

Dosage[a]	Number of dead animals per group Time after dosing (days) 1	2	3	4	5	6	7	Total[b]	Cummulative Total[c]
0	0	0	0	0	0	0	0	0/6	0
6	1	0	0	0	0	0	0	1/6	1
8	1	0	0	0	0	0	0	1/6	2
10	0	0	2	0	0	0	0	2/6	4
12	2	0	1	2	0	0	1	6/6	10
14	0	1	2	2	1	0	0	6/6	16
16	2	0	1	1	0	0	1	5/6	21

[a]T-2 toxin dosage in mg/kg body weight.
[b]Total number of animals dead over seven days.
[c]Cummulative number of dead animal.

c. Enterohepatic recirculation of T-2 toxin and its metabolites in the male Sprague-Dawley rat

Abstract

The enterohepatic circulation of T-2 toxin and its conjugated metabolites was examined in bile-cannulated male rats. Rats intraduodenally administered tritiated T-2 toxin eliminated 44.65% and 57.25 % of the administered dose in the bile within 4 and 8 hours post dosing, respectively. The thin-layer radiochromatographic metabolite profiles in the bile following intravenous and intraduodenal administration of T-2 toxin were similar. The major metabolites detected were 3'OH HT-2, glucuronide conjugates, T-2 tetraol (TOL), 4-deacetylneosolaniol (4-DN) and HT-2. Tritium-labeled glucuronide conjugates obtained from the bile of rats intravenously administered [3H]T-2 toxin were extracted and purified using C-18 and silica column chromatography. The rats eliminated 6.01% and 11.86% of the dose in the bile withinin 4 and 8 hours, respectively, following intraduodenal administration of the glucuronide conjugates. No free metabolites of T-2 toxin were detected in the bile of any animals administered the purified conjugates. Oral treatment of the rats with the ß-glucuronidase inhibitor saccharic acid lactone did not produce a statistically significant decline in the amount of radioactivity recovered in the bile following administration of the glucuronide conjugates.

Introduction

The trichothecenes are tetracyclic sesquiterpenoids containing a six-membered oxygen ring, an epoxide in the 12,13 position and an olefinic bond in the 9,10 position (Figure II.21). They are produced by a variety of fungal genera including Fusarium, Stachybotrys, and Myrothecium.

Mycotoxicoses attributed to the consumption of food naturally contaminated with trichothecenes have been reported on a worldwide scale (Ueno et al., 1971; Joffe, 1971). Adverse signs in domestic and laboratory animals associated with the consumption of trichothecene contaminated foodstuffs include: reduced weight gains, feed efficiency and feed consumption, emesis, oral lesions, diarrhea, immune suppresion, gastrointestinal irritation, abortion, cardiovascular collapse, shock and death (Bamburg and Strong, 1971, Hsu, et. al., 1972, Palyusik and Koplik-Kovacs, 1975, Speers, et. al., 1977, Weaver, et. al., 1977, Weaver, et. al., 1978a, Weaver, et. al., 1978b, Raffoni and Tuboly, 1982, Hoerr, et. al., 1982, Obara, et. al., 1984, Corrier and Zirpin, 1986).

T-2 toxin is one of the more toxic metabolites produced by species of Fusaria and has been detected as a natural contaminent of human and animal food (Bamburg and Strong, 1971; Pathre and Mirocha, 1977). T-2 toxin is rapidly eliminated from animals administered this mycotoxin with plasma disappearance half-lives ranging from 5.3, 13.8 and 17.4 in dogs, swine and cattle, respectively (Beasley et al., 1986, Sintov et al., 1986). Little parent compound is excreted intact, elimination resulting primarily through extensive and rapid biotransformation. Biotransformation of T-2 toxin occurs by four different competing pathways including ester hydrolysis at the C-4,

C-8 and C-15 positions, conjugation with glucuronic acid, aliphatic hydroxylation of the C-3' and C-4' carbon atoms on the isovaleryl side chain and by reduction of the 12, 13 epoxide (Swanson and Corley, 1988; Knupp et al., 1987b).

Enterohepatic circulation of endogenous compounds, drugs and xenobiotics is well documented. Glucuronidase activity in the intestinal contents may allow the hydrolysis of esters of ß-glucuronic acid and resorption of the liberated aglycones. Marselas et al., (1975) reported that glucarolactone shortened the pharmacological action of drugs excreted via the bile as glucuronide conjugates. They suggested that the reduced biological activity of phenobarbital and progesterone upon treatment with saccharolactone was caused by inhibition of bacterial ß-glucuronidase activity. This enzymatic inhibition would be expected to reduce the enterohepatic circulation of glucuronidated xenobiotics eliminated via the bile.

In the present study, we investigated the biliary excretion of T-2 toxin and purified glucuronide conjugates of T-2 metabolites administered intraduaodenally to male rats.

Experimental

Reference Standards

Tritium labeled T-2 toxin (500 mCi/mmol) was synthesized by New England Nuclear (Boston, MA) according to the method of Wallace et al., 1977. The tritium label was located at the C-3 position and the radiopurity was greater than 99% as determined by thin-layer radiochromatography. Unlabeled standards of T-2 toxin, HT-2, neosolaniol (NEO), 3'-OH T-2, 3'-OH HT-2, 4-deacetylneosolaniol (4-DN) and T-2 tetraol (TOL) were prepared in our laboratory as previously described (Knupp et al, 1987; Swanson et al, 1987).

Animals

Adult, male specific pathogen free Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 350-500 grams were used for all experiments. Animals were determined to be healthy by an accredited veterinarian. Animals were acclimated to the shoe-box laboratory caging for a minimum of seven days prior to each experiment. A commercially prepared rodent chow (Wayne Rodent Blocks) and deionized water were provided ad libitum. Animals were fasted overnight prior to treatment.

Surgical Preparation

Rats were anesthetized with a ketamine (6.67 mg/ml) and xylazine (66.67 mg/ml) combination administered intramuscularly at 1 ml/kg body weight. A surgical plane of anesthesia was maintained with the same drug combination for the entire experiment. Bile ducts were cannulated with PE-10 polyethylene tubing (Clay Adams, Parsippany, NJ) as descibed by Lambert,1965, and bile was collected continuously for the duration of each experiment (up to 8 hours after dosing). The body temperature was maintained with a heating pad and muscle and skin apposition. Fluid losses were compensated with intravenous administration of saline equal to the volume of bile removed.

Purification of radiolabeled conjugates from rat bile

Pooled bile was collected from bile duct cannulated rats administered tritium labeled T-2 toxin (50 Ci per rat, dissolved in 0.2 ml of 10% ethanol). Bile was collected continuously over an eight hour time period after toxin dosing. Bile from 7 rats administered tritiated T-2 toxin was pooled and an aliquot was radiochromatograped (figure 5). The pooled bile was added to a column packed with 100 g C-18 (Analytichem International, Harbor City, CA) which had been preconditioned with 200 ml of methanol followed by 200 ml of deionized water. The column was rinsed with 100 ml of water and the metabolites eluted with 600 ml of methanol. The methanol fraction was concentrated to dryness on a rotary evaporator under gentle heat ca. 45C.

The concentrated C-18 eluate was redissolved in a minimal volume of dichloromethane-methanol (2+1) and added to a 100 g silica (J. T. Baker, 70-200 mesh) column packed in dichloromethane. The silica column was washed with 420 ml of dichloromethane-methanol (3+1) followed by 635 ml of dichloromethane-methanol (1+1). One hundred ml fractions were collected and each fraction was monitored for total radioactivity by liquid scintillation counting (LSC). In addition, each fraction was monitored by thin-layer radiochromatography in order to determine metabolic profiles.

The fractions containing the suspected glucuronides, polar compounds which did not migrate form the origin of TLC plates using a mobile phase of chloroform-methanol (9+1), were combined, concentrated to dryness and stored at -20C. Prior to each experiment, the purified conjugates were redissolved in saline for administration to animals.

Analytical extraction of bile

Bile aliquots (0.5-2.0 ml) collected from the bile duct-cannulated rats administered tritium-labeled T-2 toxin, were diluted with an equal volume of water and added to 500 mg C-18 cartridges (Analytichem International, Harbor City, CA) preconditioned with methanol and water. The cartridges were washed with 2 ml of water and the metabolites eluted with 2 x 2 ml of methanol. The methanol elute was concentrated over a gentle stream of nitrogen at ca. 45°C and the residue redissolved in a minimal volume of methanol for thin-layer radiochromatographic analysis.

Thin-layer Radiochromatography

Aliquots of sample extracts were spotted onto the outer channels of activated silica TLC plates (Whatman LK5, 5 x 20 cm, 0.25 mm gel thickness). Standards of trichothecenes were spotted in the center channel. The plates were developed in chloroform-methanol (9+1) and air dried. Radioactivity profiles were determined by scraping 3 mm wide bands from the TLC plates directly into glass scintillation vials. To each vial was added 0.25 ml water, 1.0 ml of methanol and 5.0 ml Aquasol-II (New England Nuclear, Boston, MA) and the radioactivity determined by liquid scintillation counting (LSC). After scraping the bands, the plates were sprayed with 25% sulfuric acid in methanol and heated at 125C for 10 minutes in order to visualize the unlabeled standards. Selected samples of bile or

purified metabolites were incubated with either 1) acetate buffer, 2) beta-glucuronidase, or 3) beta-glucuronidase plus 1,4-saccharic acid lactone prior to thin-layer radiochromatography as described by Corley et al.,(1985).

Liquid Scintillation Counting

Bile was decolorized prior to LSC by adding aliquots (0.01 to 0.05 ml) to 7 ml glass mini vials followed by 0.10 ml of 30% hydrogen peroxide. The vials were capped and heated at 60C for 30 minutes. After cooling, 5.0 ml of Aquasol-II was added. Sample radioactivity in decolorized bile and TLC fractions were determined on a Packard 300M TriCarb liquid scintillation counter. All radioactivity data were corrected for quench, background and counting efficiency.

Beta-Glucuronidase Activity

Intestinal contents were assayed spectrophotometrically for ß-glucuronidase activity essentially as described by Fishman et al., (1967). The contents from the rat jejunum, ileum, and colon were removed and weighed, aliquots (0.5 gm) were added to 4 ml 1% Triton X and centrifuged at 2000 rpm for 15 minutes. A 0.1 ml aliquot of the supernatant fraction was added to a test tube containing 0.5 ml of phosphate buffer (0.075M, pH 6.8), 0.5 ml of phenolphthalein glucuronidate (0.0015 M, pH 7) and 0.4 ml water and incubated for 30 minutes at 37C in a water bath. Five ml of glycine buffer (0.2 M, pH 10.4) was used to stop the reaction and develop the color. A working curve was generated at each sampling with three dilutions. Absorbance was determined in a Beckman DUXX--spectrophotometer at 540 nm. Each unit of ß-glucuronidase activity was defined as the amount of enzyme which liberates one microgram of phenolphthalein from phenolphthalein glucuronide in 60 minutes at 37°C.

Experimental Protocols

Experiment #1. Seven rats were administered bile collected form rats given tritiated T-2 toxin intravenously. The animals were injected intraduodenally with 1.25 ml of donor bile containing 0.96 µCi of radiolabeled metabolites. The injection was made at the level of the duodenal ligament with a 30 gauge needle. Bile from collected continuously from the cannulated bile ducts, with the collection containers changed at two hour intervals for a total of 8 hours.

Experiment #2. Thirteen rats were injected with 0.50 ml of tritiatied T-2 toxin (1/9 v/v ethanol/saline) at three dosage levels (0.09 nmol, 5.86 nmol and 18.37 nmol). The toxin was administered intraduodenally at the level of the duodenal ligament with a 30 gauge needle. Bile was collected continuously with sample containers changed at one hour intervals for a total of 8 hours.

Experiment #3. Twelve rats were administered purified tritium labeled glucuronide conjugates (0.57 µCi dissolved in 0.50 ml water) intraduodenally as described for experiment #1. Six of the rats were treated with D-glucaro-1,4-lactone (1.2 mg/kg) by oral gavage at 3 and 6 hours prior to dosing (Group 1). Control rats (Group 2) were gavaged with an equivalent volume of water, as in group 1.

Statistical Evaluation

Analysis of variance (ANOVA) using the arc sin transformation of the percentage of the recovered radioactivity in the bile/g liver weight was used with dose being the dependent variable. A value of P 0.05 was used to indicate significance.

Results

Bile Metabolite Profiles

Bile collected from rats administered tritium labeled T-2 toxin intravenously was subjected to normal phase thin-layer radiochromatographic analysis. The major metabolites detected in the bile were 3'-hydroxy HT-2 (49%), T-2 tetraol (10%), 4-deacetylneosolaniol (5%), HT-2 (1%) and polar material remaining at the origin (35%) of the TLC plates. The polar compounds remaining at the origin were identified as glucuronide conjugates based on the following information. Incubation with limpet ß-glucurondidase produced a significant decline in the total radioactivity remaining at the origin of the TLC plates. This decline was accompanied by the appearance of the free aglycones HT-2, 3'OH HT-2 and 4-DN. Incubation of the bile with ß-glucuronidase in the presence of saccharic acid lactone eliminated the conversion of the polar material to the free aglycones and yielded profiles which were identical to that produced by incubation of the conjugates with buffer alone.

Purification Radiolabeled Glucuronide Conjugates

A total of twenty-four ml of bile (48 µCi) was collected from seven rats intravenously administered tritium labeled T-2 toxin. A flow chart of the scheme used for purification of the radiolabeled conjugates is shown in Figure II.22. Nearly all of the radioactivity in the bile (99%) was extracted by the C-18 column and eluted in the methanol fraction. A subsequent silica column was used to separate the free T-2 toxin and its metabolites from the more polar glucuronide conjugates. All free trichothecenes metabolites were eluted with dichloromethane-methanol (3+1). The radioactivity eluting with dichloromethane-methanol (1+1) represented 37% of the initial radioactivity present in the pooled bile and consisted entirely of material which remained at the origin upon normal phase thin-layer radiochromatographic analysis using chloroform-methanol (9+1) as the developing solvent.

Confirmation of the compounds present in the silica column dichorormethane-methanol (1+1) fraction as glucuronide conjugates and identification of the aglycones was accomplished by incubation with ß-glucuronidase followed by thin-layer radiochromatography. Enzymatic hydrolysis with ß-glucuronidase resulted in the elimination of radioactivity remaining at the TLC plate origin and the corresponding appearance of radioactivity co-migrating with the following T-2 metabolites (% of total residues): HT-2 (59%), 3'OH HT-2 (17%), 4-DN (15%) and T-2 tetraol (5%). See Figure II.23 for a representative chromatograms. Upon addition of saccharic acid lactone to the incubation mixture, TLC profiles were identical to

incubation of the conjugates with buffer alone, all radioactivity remained at the origin of the TLC plates.

Experiment #1. A total of 8.89% ± 1.64 and 15.03% ± 3.43 of the dose was recovered in the bile within 4 and 8 hours, respectively, after intraduodenal administration of tritiated donor bile to the recipient rats. See Table II.10.

Experiment #2. The time course for the elimination of total radioactivity in the bile of rats intraduodenally administered three different dosages of T-2 toxin is shown in Figure II.24. There was a steady increase in radioactivity eliminated in the bile over the 8 hour experimental time period. Profiles were similar for all three dosages and there was no significant differences between the mean values of the three groups. The values for all three dosages at each time point were therefore pooled. A total of 44.65% 1.97 and 57.25% 1.87 of the dose (total radioactivity) was recovered in the bile by 4 and 8 hours, respectively.

The metabolites present in the bile of rats administered tritium labeled T-2 intaduaodenally were identified by thin-layer radiochromatography. The major compounds detected were (mean standard deviation) 3'OH HT-2 (53%), T-2 tetraol (17%), 4-deactylneosolaniol (6%), HT-2 (1%) and polar material remaining at the origin (23%). See Figure II.25 for a representative radiochromatogram.

Experiment #3. In Experiment #3, rats were dosed intraduodenally with purified tritium-labeled glucuronide conjugates and the bile was collected over an eight hour total time period. A total of 6.01% ± 0.80 and 11.86% ± 1.47 of the dose was recovered in the bile of these rats by 4 and 8 hours, respectively. See Table II.10.

A second group of rats were administered the purified conjugates in addition to oral treatment with the ß-glucuronidase inhibitor saccharic acid lactone. Although less of the administered dose was recovered at 4 hours (4.91% 0.06) and 8 hours (8.12% 1.36) in the bile compared to untreated control rats recieving conjugates only (Table II.10), the difference was not statistically different.

Metabolic profiles of the bile by radiochromatography were similar in nature between these two groups and consisted entirely of polar compounds which did not migrate from the origin of the TLC plates using the solvent system employed. A representative radiochromatogram of bile collected from rats administered the purified glucuronide conjugates is given in Figure II.25.

Treatment of the rats with saccharic acid lactone completely inhibited ß-glucuronidase activity in the intestinal contents obtained from both the jejunum and ileum. Although the inhibition of ß-glucuronidase activity was less pronounced in the large compared to the small intestine, activity was still reduced by 92.4% compared to animals recieving the conjugates alone. See Table II.11 for a summary of the data.

Discussion

Glucuronide conjugates of T-2 toxin and related trichothecenes such as diacetoxyscirpenol (DAS) and deoxynivalenol (DON) have been demonstrated to be major elimination products in a variety of species (Gareiss et al., 1986; Corley et al., 1986; Côté et al., 1986; Pace et al., 1986). Although the enterohepatic recirculation of T-2 toxin and its metabolites has been suggested, data supporting this conclusion was limited. In the present study we demonstrated that both free metabolites of T-2 toxin and their glucuronide conjugates are recycled in the rat.

T-2 metabolites are enterohepatically circulated in the male rat. The protracted effects ascribed to T-2 toxicosis may be attributed to this metabolic recirculation.

The parent compound was not detected in any of the experiments. The rapid biotransformation of T-2 toxin has previously been reported in many species. The metabolic profile of bile was similar when rats were dosed with tritiated T-2 toxin intravenously or intraduodenally (3'-hydroxy HT-2 is the major metabolite). Approximately a quarter (ID) to one-third (IV) of the administered dose was converted to glucuronide conjugates in the rat. Intraduodenal administration of tritium labeled T-2 toxin had no effect on the absorption and elimination of T-2 toxin over the range of administered dose, 0.09-18.37 nmol.

Twelve percent of the intraduodenally administered glucuronide conjugates was eliminated in the bile within eight hours of dosing. Radiochromatography of bile from all rats dosed with conjugates showed no migration of the radioactivity. This may suggest that a threshold for glucuronidation exists in the rat. Pretreatment with saccharolactone did not significantly change the biliary elimination of intraduodenally administered glucuronide conjugates. This may indicate that hydrolysis of the conjugates by intestinal ß-glucuronidase is not necessary for the absorption and elimination of T-2 toxin conjugates in the bile of rats.

Acknowledgement

The technical assistance of Barb Kindler, Tina Kerferlis, Nada Little and Dick Manual was greatly appreciated.

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Table II.11 Percent of radioactivity excreted in the bile of rats administered T-2 toxin, donor bile from T-2 toxin treated rats or purified glucuronide conjugates intraduodenally.

Compound	N	% of administered dose	
		4 hours[a]	8 hours
T-2 toxin	13	44.65 ± 1.97[b]	57.25 ± 1.87
Donor bile[c]	7	8.89 ± 1.64	15.03 ± 3.43[d]
Conjugates[e]	6	6.01 ± 0.80	11.86 ± 1.47
Conj + INH[f]	6	4.91 ± 0.96	8.12 ± 1.36

[a]Time after administration.

[b]Mean standard error of the mean.

[c]Bile obtained from rats administered tritium-labeled T-2 toxin intravenously.

[d]N = 4.

[e]Purified conjugates of T-2 metabolites. See Figure II.21 for aglycone composition.

[f]Animals administered purified glucuronide conjugates of T-2 metabolites and orally treated with saccharolactone (1.2 mg/kg bw at 3 and 6 hours pre-dosing).

Table II.12 Inhibition of ß-glucuronidase activity in the intestinal contents of rats administered glucuronide conjugates of T-2 toxin metabolites following treatment with the enzyme inhibitor saccharic acid lactone.

Gut Segment	B	Units ß-Glucuronidase Act[a]		
		CONJ + INH[b]	CONJ[c] %	INH[d]
Jejunum	6	0 ± 0[e]	67.1 ± 11.3	100.0 ± 0
Ileum	6	0 ± 0	570.9 ± 44.5	100.0 ± 0
Colon	6	161.7 ± 16.1	2136.0 ± 427.4	92.4 ± 0.7

[a]One unit of ß-glucuronidase acitivity is defined as the activity required to liberate one µg of phenolphthalein from phenolphthalein glucuronide in one hour at 37°C.

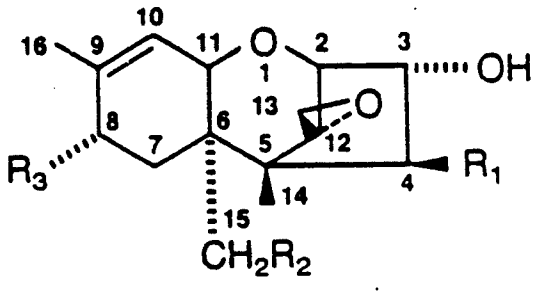
[b]Animals pretreated with saccharolactone orally and administered glucuronide conjugates.

[c]Animals receiving conjugates only.

[d]Percent inhibition of ß-glucuronidase in rats recieving saccharolactone relative to rats receiving conjugates only.

[e]Mean ± standard error.

Figure II.21 Structures of T-2 toxin and related metabolites.



Compound	R1	R2	R3
T-2	OAc	OAc	ISV
HT-2	OH	OAc	ISV
4-DN	OH	OAc	OH
TOL	OH	OH	OH
TRIOL	OH	OH	ISV
3'OH T-2	OAC	OAc	ISV-OH_2
3'OH HT-2	OH	OAc	ISV-OH_2

ISV = $OOCCH_2CH(CH_3)_2$
ISV-OH = $OOCCH_2COH(CH_3)_2$

Figure II.22 Flow chart for the extraction and purification of the glucuronide conjugate fraction of T-2 toxin metabolites from the bile of rats intravenously administered tritium-labeled T-2 toxin.

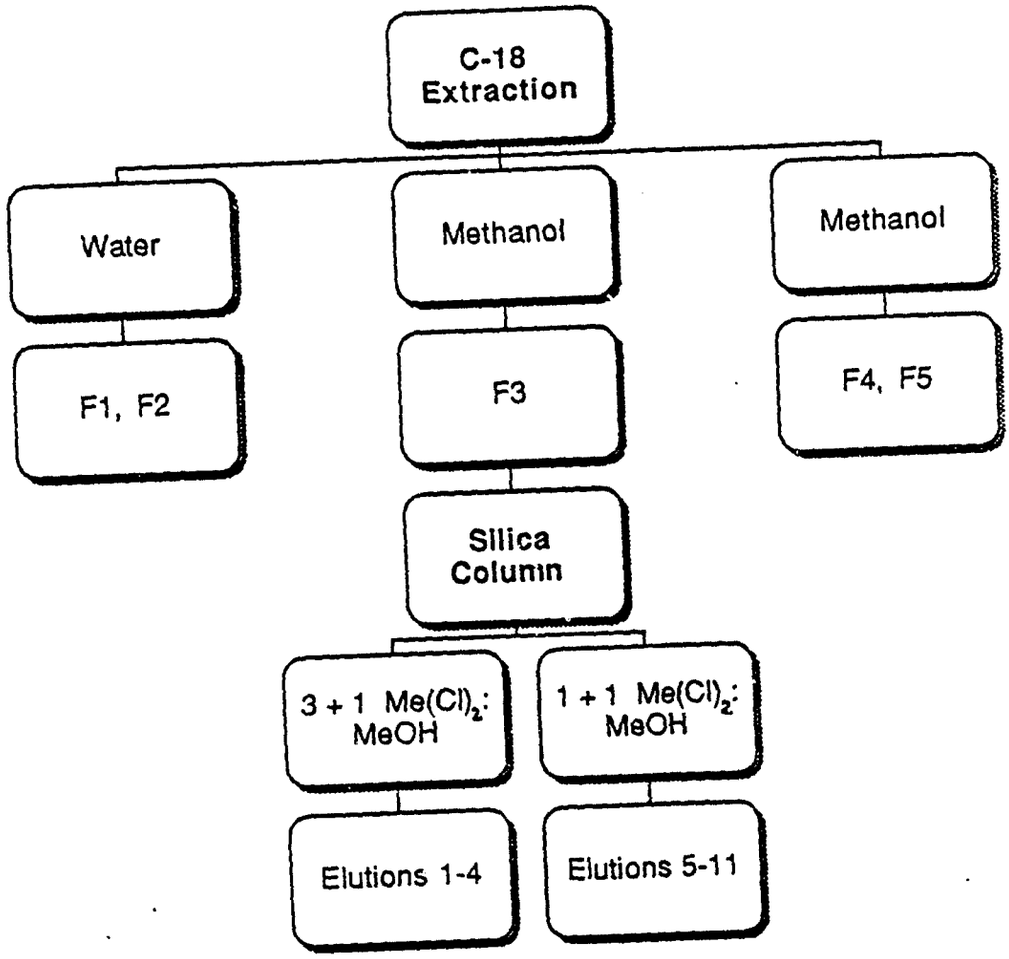


Figure II.23 Thin-layer radiochromatograms of purified bile conjugates incubated with A) buffer only, B) buffer, ß-glucuronidase and saccharolactone, C) buffer and ß-glucuronidase.

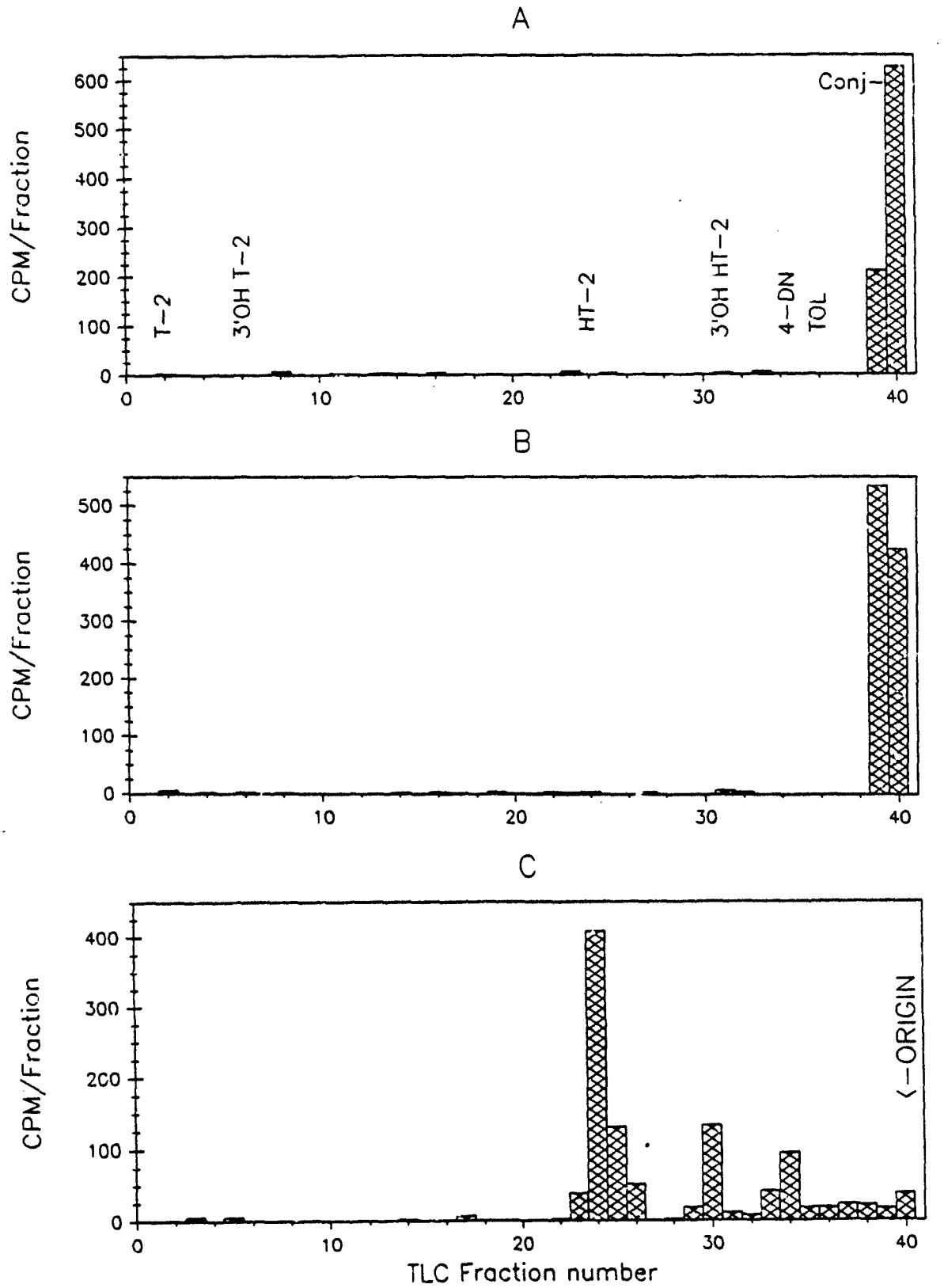


Figure II.24 Time course of the radioactivity in the bile after intraduodenal administration of tritiated T-2 toxin. The cumulative amount excreted into the bile is given as a percent of the administered dose. The mean values are given from three experiments, with a group size of at least four animals. The standard error bars have been deleted from the middle graph for the sake of clarity. No significant difference was seen between the dosage groups using a student's t-test ($P > 0.05$).

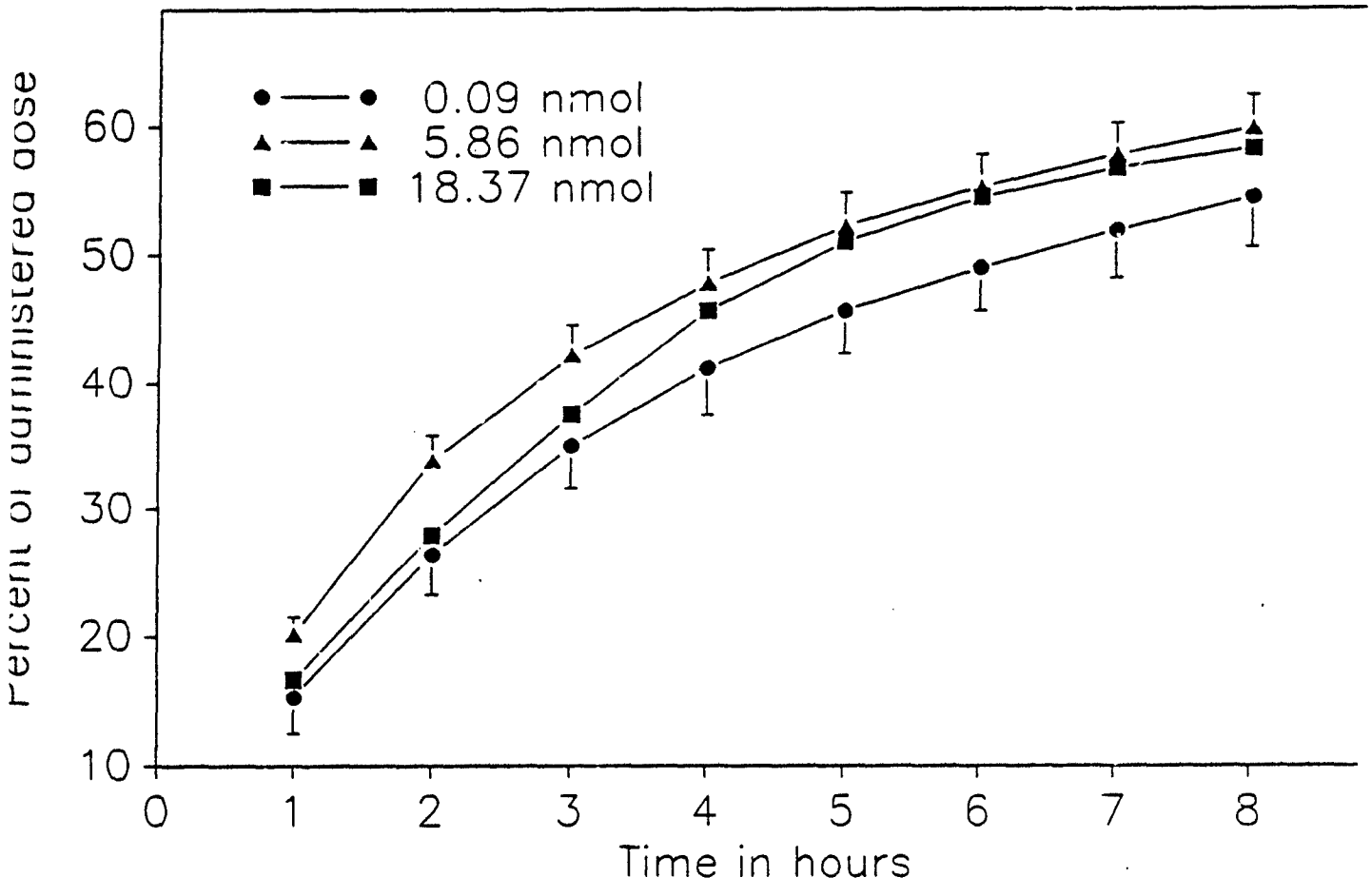
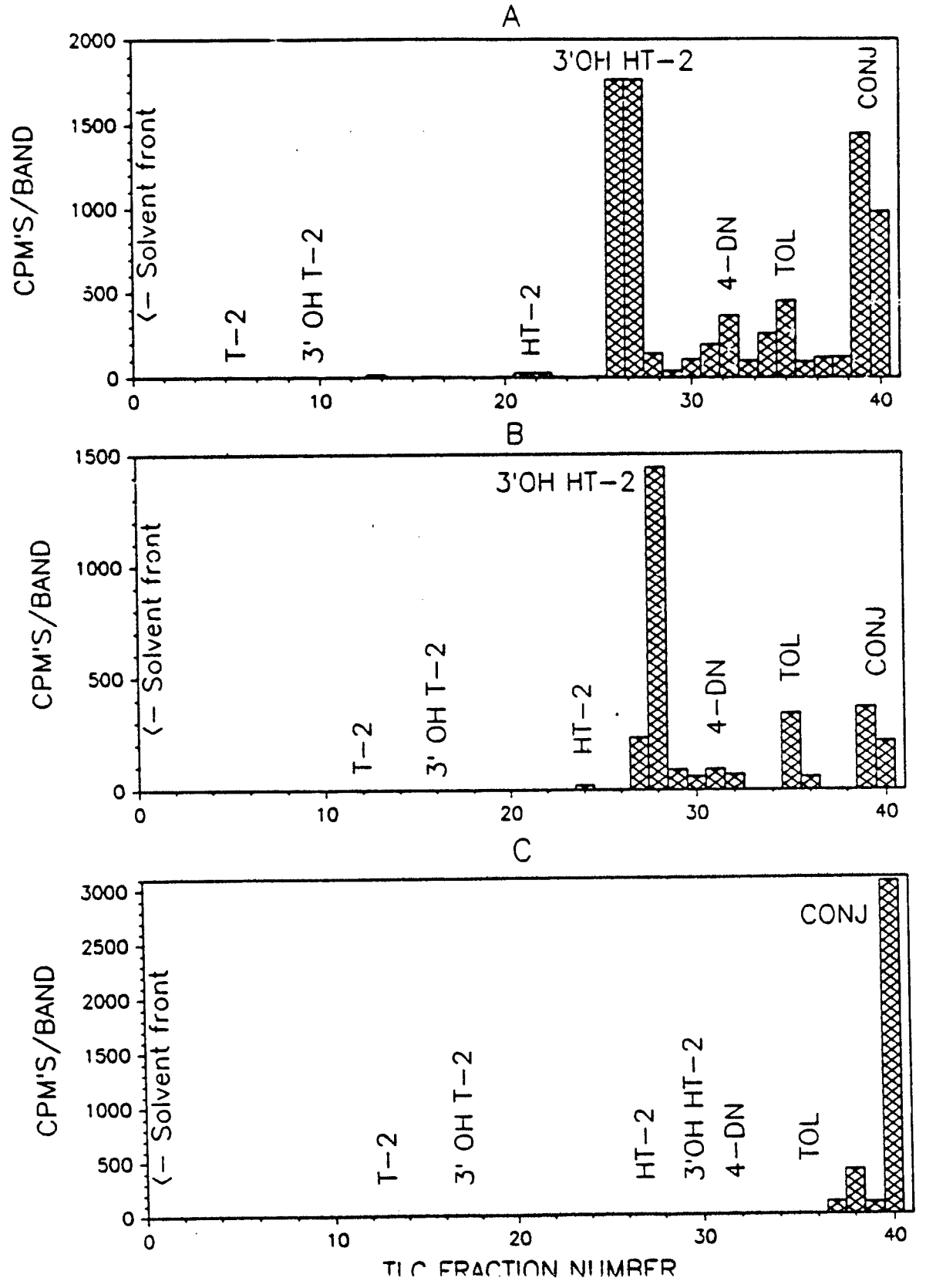


Figure II.25 A. Thin-layer radiochromatogram of bile from rats administered T-2 toxin intraduodenally.
B. Thin-layer radiochromatogram of bile from rats administered T-2 toxin intravenously.
C. Thin-layer radiochromatogram of bile from rats administered purified glucuronide conjugates intraduodenally.



d. Metabolism of T-2 toxin in rabbits: effects of dose, route, and time

Abstract

Metabolic and elimination profiles were investigated in rabbits following intavenous, dermal and oral administration of tritium-labeled T-2 toxin at 0.15 and 0.6 mg/kg body weight. The major metabolites detected in the urine were T-2 tetraol (TOL), deepoxy T-2 tetraol (DE TOL), 3'OH HT-2 and an unknown metabolite labeled M9. In feces, the major products eliminated were HT-2, 3'OH HT-2, DE TOL, and M9. The metabolite M9 was tentatively identified as deepoxy 3'OH HT-2. There were significant effects due to dose and route, but the effect of time was much more significant. The percentage of deepoxy metabolites compared to the total residues detected, increased steadily over time with all three routes of administration and both dosages investigated.

Introduction

The trichothecene mycotoxins are a group of fungal metabolites characterized by a 12, 13-epoxytrichothec-9-ene skeleton. T-2 toxin, one of many toxic fungal metabolites, is produced primarily by species of Fusarium (Bamberg and Strong, 1971). T-2 toxin has been implicated in a variety of toxicoses including moldy corn toxicosis, bean-hull poisoning and alimentary toxic aleukia (Hsu et al., 1972; Puls and Greenway, 1976; Ueno et al., 1972; Joffe, 1971).

T-2 toxin is rapidly metabolized and eliminated in a feces to urine ratio of 3:1 in mice, 5:1 in rats and 1:4 in guinea pigs (Matsumoto et al., 1978; Pace et al., 1985).

Metabolism studies using both *in vivo* and *in vitro* systems indicated that the metabolism of T-2 toxin can proceed by four different pathways. These metabolic pathways included hydrolysis, hydroxylation, deepoxidation and glucuronidation. The hydrolysis of T-2 toxin to T-2 tetraol via several intermediates has been reported using rat liver homogenates (Ohta et al., 1978; Yoshizawa et al., 1980). Cytochrome P450 catalyzed hydroxylation at the C-3' position of HT-2 and T-2 toxin has been demonstrated using liver homogenates from mice and monkeys and glucuronidation as a primary metabolic pathway has been reported for swine (Yoshizawa et al., 1985; Pfeiffer et al. manuscript in preparation).

The major metabolites in the excreta of pigs, cattle, chickens, rats and guinea pigs included 3'OH HT-2, HT-2, 4- deacetylneosolaniol, T-2 tetraol and 3'OH T-2 (Corley et al., 1985; Pace et al., 1985; Yoshizawa et al., 1980, 1981 and 1982; Visconti and Mirocha, 1982; Pfeiffer et al., manuscript in preparation). Qualitatively, there appear to be few species differences in the metabolism of T-2 toxin.

In a previous study using rats, the effects of dose, route and time on the metabolism of T-2 toxin were examined (Pfeiffer et al., manuscript in preparation). The purpose of this study was to examine these effects in a nonrodent species, namely; the rabbit.

Experimental

Reference Standards

Tritium-labeled T-2 toxin (labeled in the C-3 position, radiopurity > 97%, 500 mCi/mmole) was obtained from Amersham Corporation, Arlington Heights, Il. Unlabeled standards of T-2 toxin, neosolaniol, HT-2, T-2, triol, 4-deacetylneosolaniol and T-2 tetraol were produced from cultures of Fusarium sporotrichioides and purified in our laboratory as previously described (Swanson et al., 1987b; Knupp et al., 1987).

Tritium-labeled standards of HT-2, T-2 triol and T-2 tetraol were prepared by alkaline hydrolysis of [3] T-2 toxin (Wei et al., 1971). Tritium-labeled 3'OH T-2 toxin was prepared by incubation of labeled T-2 toxin with S-9 rat liver homogenates as described by Knupp et al., 1987b. Radiolabeled standards of 3'OH HT-2 and 3'OH T-2 triol were prepared by mild alkaline hydrolysis of radiolabeled 3'OH T-2 toxin (Wei et al., 1971). Radiolabeled deepoxy derivatives including deepoxy HT-2 (DE HT- 2), deepoxy T-2 triol (DE TRIOL) and deepoxy T-2 tetraol (DE TOL) were prepared by incubating tritium-labeled T-2 toxin with bovine rumen microflora under anaerobic conditions (Swanson et al., 1987a).

Confirmation of these metabolites was done by comparison with reference standards using thin-layer and gas-liquid chromatography.

Animal Treatment

Male, New Zealand White rabbits weighing 700 to 1100 grams (5-6 weeks old) were obtained from Lesser's Rabbitry, Union Grove, WI. The animals were housed individually in metabolism cages and acclimated to a 12-hour day-night cycle for seven days prior to dosing. Free access to water and a two-hour feeding time were used except for 12 hours predosing when both were removed. Urine and feces were collected for six days after which the rabbits were killed by intracardiac injection of sodium pentobarbital.

Animal Dosing

Each rabbit was given 200 µCi tritium-labeled T-2 toxin at either 0.15 or 0.60 mg/kg of body weight. Intravenous doses were administered in 0.25 ml of 50% ethanol/water through an ear vein. Oral doses were dissolved in 0.25 ml of 50% ethanol/water and administered by gelatin capsule. Dermal doses were applied to an area approximately 1 cm located between the scapulas in 0.1 ml of 90% DMSO/water. Each route and dose combination was repeated 3 times for a total of 18 rabbits. Feces and urine were collected every six hours and stored at -20°C until used for analysis.

Determination of Total Radioactivity

The total radioactivity in urine was determined by adding 0.1 ml plus 0.4 ml water directly to 5 ml Aquasol-2 liquid scintillation cocktail (New England Nuclear Corp., Boston, MA). The total radioactivity in feces was determined by first homogenizing 1.0 gram in 20 ml of 0.1 M acetate buffer (pH 3.8). A 0.1 ml aliquot was removed and added to a 7 ml glass vial containing 0.1 ml perchloric acid (60%) and 0.2 ml

hydrogen peroxide (30%). The vials were capped tightly and heated to 60°C for 24 hours. Following heating, the vials were allowed to cool, and 5 ml Aquasol-2 was added. Urine and feces samples were counted on a Packard Tri-Carb 300M Liquid Scintillation Counter (Packard Inst., Chicago, IL).

Urine Extraction

A 0.1 to 10.0 ml volume depending on the amount of total radioactivity present was diluted to 15 ml with water, followed by the addition of 5 g NaCl and 15 ml acetonitrile. Upon centrifugation at 2000 rpm, the top layer was removed. The extraction was repeated 3 times with acetonitrile and a fourth time with acetonitrile/acetone (1 + 1). The top layers were combined, and 100 ml of methylene chloride was added to drive residual water out of solution. Anhydrous sodium sulfate (approximately 50 g) was added to remove water (solutions appeared clear). Cupric carbonate (2.5 g) was added, and the samples were filtered through ash-free analytical filter pulp (Schleicher and Schuel, Inc., Keene, NH). The pulp and flasks were rinsed 3 times with 20 ml ethyl acetate. Samples were then concentrated to dryness and redissolved in 1 to 2 ml methanol, diluted with an equal volume of water and filtered through a disposable membrane filter (Arco LC25, 0.2 micron; Gilman Sciences, Ann Arbor, MI) for HPLC radiochromatographic analysis.

Feces Extraction

To a 0.5 to 3.0 g sample was added 10 ml of 0.1 M acetate buffer (pH 3.8) and 10 ml acetonitrile. Each sample was mixed well and centrifuged at 2000 rpm and the aqueous acetonitrile layer was transferred to a 30 ml plastic centrifuge tube. The feces were extracted 3 times with 10 ml acetonitrile/water (1 + 1). To the combined aqueous acetonitrile portions was added 5 g NaCl followed by shaking and centrifugation to allow the phases to separate. The acetonitrile layer was removed and the extraction was repeated 3 times with 10 ml acetonitrile and a fourth time with acetonitrile/water (1 + 1). The acetonitrile extracts were diluted with methylene chloride and processed as described above for urine.

HPLC Radiochromatography

An HPLC system (Perkin Elmer Series 4, Norwalk, CT) was equipped with a 15 cm x 4.6 mm id column packed with 5 micron C18 (Econosphere, Alltech Assoc., Deerfield, IL). A linear mobile phase gradient from 20 to 90% methanol in water over 30 minutes at a flow rate of 1.0 ml/min was used to separate metabolites. Fractions (0.2 ml) were collected and the radioactivity was determined by liquid scintillation counting in Scinti Verse LC liquid scintillation cocktail (Fisher Scientific Co., Itasca, IL).

Thin-Layer Radiochromatography

Aliquots of samples were spotted into the outer channels of a precoated silica gel TLC plate (5 x 20 cm, 0.25 mm gel thickness, J. T. Baker) which was activated for 1 hour at 110°C. Plates were developed in chloroform/methanol (9 + 1) and allowed to air dry. To obtain radiochromatographic profiles, 1 to 2 mm bands were scraped

from the TLC plate directly into scintillation vials. A 0.1 ml volume of water was added to each vial, followed by 0.25 ml methanol. Samples were then counted in 5 ml Aquasol-2 . Standards were visualized under long wave (365 nm) UV lamp after having been sprayed with 30% sulfuric acid in methanol and heated at 110°C for 35 min.

Statistical Analysis

Analysis of variance (ANOVA) using the percentage of extracted radioactivity (perofext) as the dependent variable was used. A value of $p < 0.05$ was used to indicate significance. Tukey's Studentized Range test ($p < 0.05$) was used for comparisons between route, dose and time.

Results

Total Radioactivity in Urine and Feces

The total cumulative and daily excretion of radioactivity in rabbit urine and feces are shown in Figures II.26 to II.30. The ratio of radioactivity in feces compared to urine was approximately 1:2 over all treatment combinations. The excretion of radioactivity was rapid and after 72 hours, 70 to 80% of the radioactivity was excreted in i.v. and orally dosed rabbits, but only 30 to 50% in dermally dosed rabbits. The excretion of radioactivity was nearly complete (> 95%) in i.v. and orally dosed rabbits after six days, but only 65% of the radioactivity was excreted in dermally dosed rabbits.

Metabolite Determination

The percent of radioactivity extracted (perofext) in urine and feces was 36.7 $\pm$ 2.2 and 45.1 $\pm$ 1.5 ($\bar{x} \pm SE$, $n = 54$), respectively. No differences were noted in the extraction efficiencies between doses and routes, but a slight decrease over time was observed.

The methodology developed for this study was well suited for the analysis of tritium-labeled T-2 toxin and metabolites, which have a wide range of polarities (Pfeiffer et al., manuscript in preparation). The structures of T-2 toxin and several metabolites are given in Table II.13.

HPLC analysis of urine and fecal extracts resulted in 15 different radioactive peaks. A total of 72.5% of the extracted radioactivity in urine averaged over dose, route and time had HPLC retention times identical to standards of T-2 toxin (1.6%), deepoxy T-2 tetraol (16.1%), T-2 tetraol (25.8 percent) and 3'OH HT-2 (29.2%). Two unknown metabolites labeled M3 (5.0%) and M9 (13.0%) accounted for an additional 18.0% of the extracted radioactivity in urine extracts. In a previous study in rats, a radioactive peak having the same HPLC retention time as M9 (unknown metabolite) was tentatively identified as deepoxy 3'OH HT-2 (Pfeiffer et al., manuscript in preparation). The HPLC retention times for the 15 major radioactive peaks are shown in Table II.14.

In feces, a total of 59.9% of extracted radioactivity averaged over dose, route and time had HPLC retention times identical to deepoxy HT-2 (4.6%), T-2 tetraol (5.4%), HT-2 (13.3%), 3'OH HT-2 (17.1%) and

deepoxy T-2 tetraol (19.5%). Unknown metabolite M9 accounted for another 26.0% of the extracted radioactivity.

The metabolic profiles (HPLC radiochromatograms) for representative urine and fecal extracts are shown in Figure II.31. The metabolic profiles in urine and feces, by route and dose, expressed as a percent of the extracted radioactivity over time are given in Tables II.15 to II.17 and the total metabolic profile summed over days 1, 2 and 3 and expressed as a percent of the administered radioactivity is given in Table II.18.

Statistical Interpretation for Urine

The main effect identified in urine extracts was time. The effects of time for the major metabolites in urine are shown in Figure II.32. For the deepoxy metabolites, deepoxy T-2 tetraol and M9, the percentages for day 1 are significantly less than for days 2 or 3. For T-2 tetraol, 3'OH HT-2, HT-2 and T-2 toxin, the percentages for day 1 were significantly greater than for days 2 and 3.

The effects of dose and route were present but only for a few major metabolites in urine as shown in Figures II.32 to II.33. The percentage of T-2 tetraol in urine extracts from the high dose group was greater than the low dose group. The percentages of T-2 toxin and unknown metabolite M3 were significantly greater in dermally dosed rabbits. Interactions did not play an important role in the statistical analysis, due in part, to the overwhelming effect of time.

Statistical Interpretation for Feces

As with urine, time was the most significant effect noted in feces. The percentages of deepoxy metabolites were greater on days 2 or 3, whereas other metabolites in fecal extracts such as HT-2 and T-2 toxin, the percentages were greatest on day 1. In extracts from rabbit feces, HT-2 accounted for 40 percent of the extracted radioactivity on day 1 , but then declined rapidly to 30% on day 2 and 1.0% on day 3.

The effects of dose and route were significant only for metabolite M9, deepoxy HT-2 and T-2 toxin. The percentages of M9 and deepoxy HT-2 were greater for the low dose group of rabbits. The percentage of HT-2 was significantly greater for i.v. dosed rabbits, but for T-2 toxin, the percentage was significantly greater for dermally dosed rabbits than either orally or i.v. dosed rabbits. The results of the statistical analysis for feces are shown in Figures II.34 to II.36.

Discussion

The rapid excretion of T-2 toxin and metabolites in this study was consistent with that reported for other species such as rats, chickens, guinea pigs, and cattle (Matsumoto et al., 1978; Pace et al., 1985; Yoshizawa et al., 1980, 1981). The urine to feces ration of radioactivity in the rabbit was 2:1. This varies from the rat (1:4), but is similar to the guinea pig (4:1). This may be attributed to differences in the molecular weight thresholds for biliary excretion, which is estimated to be 325, 440, and 475 amu for the rat, guinea pig and rabbit, respectively (Calabrese, 1983).

The slower rate of excretion following topical application has been reported in other species. In swine, the skin and surrounding fat have been shown to be a reservoir for T-2 toxin and a site of metabolism (Pang et al., 1987).

The slower rate of excretion of radioactivity in the high dose dermnal rabbits compared to the low dose group may be due to the more extensive tissue damage observed in the high dose group resulting in a slower rate of absorption.

Chromatographic analysis of the radioactivity in the excreta of rabbits, regardless of dose or route, identified the major metabolites as T-2 tetraol, 3'OH HT-2 and HT-2. These metabolites have also been reported in cattle, swine, chickens, guinea pigs, and rats which indicated no qualitative species differences in the metabolism of T-2 toxin. Also significant in the excreta of rabbits were deepoxy T-2 tetraol and metabolite labeled M9, which was tentatively identified as deepoxy 3'OH HT-2 (Yoshizawa et al., 1985). The significant occurrence of deepoxide metabolites in the excreta of rats has also been reported (Pfeiffer et al., submitted for publication).

The results of this study indicated that the metabolism of T-2 toxin in rabbits proceeded by three distinct pathways: hydrolysis, hydroxylation and deepoxidation. This is in agreement with in vivo and in vitro metabolism studies in rats, chickens, cattle, swine, and guinea pigs (Yoshizawa et al., 1980, 1981, 1984, 1985; Pace et al., 1985; Corley et al., 1985).

The glucuronidation pathway reported in swine was not investigated in this study (Corley et al., 1985). However, the decreased extraction efficiency over time may indicate the involvement of another pathway which results in more polar metabolites such as conjugates and/or more extensive binding over time. The majority of unextracted radioactivity was polar in nature and remained at the origin of TLC plates developed in chloroform-methanol (9+1). Although unconclusive, this data is consistant with the conclusion that the unextracted radioactivity was composed of glucuronide conjugates of T-2 toxin or its metabolites.

The deepoxy metabolites accounted for a significant percentage of the metabolites both as a percent of the extracted radioactivity and as a percent of the dose (see Tables II.15 to II.18). Toxicologic data suggest that deepoxidation of trichothecenes results in detoxification. Three deepoxy metabolites of T-2 toxin, deepoxy HT-2, deepoxy T-2 triol and deepoxy T-2 tetraol displayed no toxicity to brine shrimp (Artemia salena) at concentrations up to 5000 ng/ml (Swanson et al., 1987b). In addition, deepoxy T-2, displayed no toxicity to mice administered up to 60 mg/kg bw intraperitoneally and was at least 400 times less toxic than T-2 toxin in the rat skin irritation bioassay (Swanson et al., submitted for publication).

Statistical analysis indicated that time was the most significant effect on the metabolic profiles in fecal extracts from rabbits. The highest percentages for deepoxy T-2 tetraol and metabolite M9 were on days 2 or 3, whereas the maximum percentages for HT-2 and T-2 toxin were on the first day. The high percentage of deepoxy HT-2 in fecal

extracts on day 1 seems to be associated with the very high percentage of HT-2 in extracts on day 1.

The effect of time was also significant for urinary metabolites. The percentages of T-2 tetraol and 3'OH HT-2 were greatest on day 1, whereas the percentages of deepoxy T-2 tetraol and metabolite M9 were greater on days 2 or 3.

The microsomal carboxyesterase activity in rabbit liver homogenates has been reported to be 1000 times greater than that in the rat (Ohta et al., 1977). Since microsomal carboxyesterases in the liver are reported to be responsible for the hydrolysis of T-2 toxin, the very high percentage of HT-2 in fecal extracts from rabbits was expected.

The effect of route was significant in urine for T-2 toxin and feces for HT-2 and T-2 toxin. In urine and fecal extracts, the percentage of T-2 toxin in dermally dosed rabbits was significantly higher than in i.v. and orally dosed rabbits. Further statistical analysis for the effect of time indicated that this increase in the percentage of T-2 toxin in dermally dosed rabbits also increased over time. This may result from the inhibition of protein synthesis by T-2 toxin reported by Ueno et al. (1973), which leads to enzyme inhibition which could result in impaired metabolic capabilities.

The percentage of HT-2 was statistically higher in i.v. dosed rabbits due possibly to the greater initial availability of T-2 toxin for hydrolysis by the liver microsomal carboxyesterase.

The effect of dose on the major metabolites was not as significant as route and time, but the effect was present. The percentages of deepoxy HT-2 and M9 were higher in fecal extracts from low dose rabbits, whereas T-2 tetraol was higher in urine extracts from high dose rabbits. For HT-2 and M9 in fecal extracts and T-2 tetraol in urine extracts, the dose/time interaction was significant. The significant effect of time was primarily responsible for this interaction.

Enterohepatic recirculation of T-2 toxin and/or metabolites has been discussed by several authors (Corley et al.,1985; Yoshizawa et al., 1981). Deepoxidation of T-2 toxin has been reported to occur only in the intestinal tract of rats (Yoshizawa et al., 1985). As a result, the presence of significant percentages of deepoxy metabolites, especially deepoxy T-2 tetraol in urine, tends to support this hypothesis.

The results of this study indicated that deepoxidation is an important metabolic pathway for T-2 toxin in rabbits. Since the toxicities of the deepoxy metabolites are condsiderably less than the other metabolites of T-2 toxin, their formation is an important detoxification pathway. Changes in the intestinal microflora that may inhibit this pathway or the lack of the specific microflora could lead to a greater toxic effect following exposure to T-2 toxin.

The microsomal carboxyesterase activity in rabbit liver homogenates has been reported to be 1000 times greater than that in the rat (Ohta et al., 1977). Since microsomal carboxyesterases in the liver are

reported to be responsible for the hydrolysis of T-2 toxin, the very high percentage of HT-2 in fecal extracts from rabbits was expected.

The effect of route was significant in urine for T-2 toxin and feces for HT-2 and T-2 toxin. In urine and fecal extracts, the percentage of T-2 toxin in dermally dosed rabbits was significantly higher than in i.v. and orally dosed rabbits. Further statistical analysis for the effect of time indicated that this increase in the percentage of T-2 toxin in dermally dosed rabbits also increased over time. This may result from the inhibition of protein synthesis by T-2 toxin reported by Ueno et al. (1973), which leads to enzyme inhibition which could result in impaired metabolic capabilities.

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Acknowledgement

This project was supported in part by the U.S. Army Medical Research and Development Command, Contract no. DAMD 17-85-C-5224.

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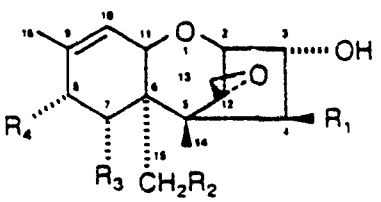
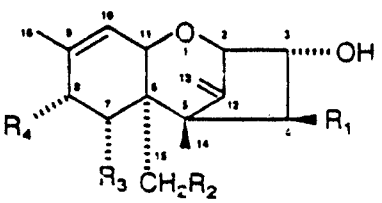
Table II.13 Chemical structures of T-2 toxin and selected metabolites.

Name	R1	R2	R3	R4	Ring
T-2 toxin	OH	OAc	OAc	ISV	A
3'OH T-2	OH	OAc	OAc	ISV-OH	A
Neosolaniol	OH	OAc	OAc	OH	A
HT-2	OH	OH	OAc	ISV	A
3'OH HT-2	OH	OH	OAc	ISV-OH	A
T-2 triol	OH	OH	OH	ISV	A
T-2 tetraol	OH	OH	OH	OH	A
DE 3'OH HT-2	OH	OH	OAc	ISV-OH	B
DE HT-2	OH	OH	OAc	ISV	B
DE T-2 tetraol	OH	OH	OH	OH	B

ISV = $OCOCH_2CH(CH_3)_2$; ISV-OH = $OCOCH_2COH(CH_3)_2$



A.



B.

Table II.14 Retention times of the fifteen radioactive peaks by HPLC.

Metabolite	RT (min)
T-2 toxin	24.2
de HT-2	23.6
HT-2	21.8
de Triol	20.6
M12	19.8
Triol	19.4
3'OH T-2	18.2
M9	16.8
3'OH HT-2	14.6
M7	13.4
M6	12.2
M5	10.8
M3	9.2
de Tetraol	6.4
Tetraol	4.4

Table II.15a Metabolic profiles expressed as a percent of the extracted radioactivity in the excreta of rabbits 24 hours after administration of tritium-labeled T-2 toxin.

Metabolite	Route and Dosage: 0.15 mg/kg Oral	0.6 mg/kg Oral	0.15 mg/kg I.V.
URINE			
TOL	27.8 ± 0.7	23.7 ± 2.1	34.3 ± 1.8
deTOL	9.2 ± 3.3	7.0 ± 3.6	3.2 ± 0.7
M3	6.4 ± 0.7	5.9 ± 0.8	6.3 ± 0.8
M5	1.6 ± 0.3	2.1 ± 0.5	2.1 ± 0.5
M6	1.5 ± 0.2	1.7 ± 0.1	0.9 ± 0.01
M7	0.4 ± 0.1	15.0 ± 9.4	0.2 ± 0.01
3'OH HT-2	39.0 ± 1.9	40.6 ± 4.6	38.2 ± 1.6
M9	7.3 ± 1.7	6.4 ± 2.7	4.8 ± 1.3
3'OH T-2	ND	0.7 ± 0.4	0.8 ± 0.1
Triol	0.6 ± 0.1	2.3 ± 1.5	0.6 ± 0.3
M12	0.5 ± 0.2	0.8 ± 0.5	0.5 ± 0.1
deTriol	0.3 ± 0.1	0.5 ± 0.1	0.1 ± 0.01
HT-2	2.7 ± 1.3	6.6 ± 1.0	7.2 ± 1.3
deHT-2	ND	0.2 ± 0.2	ND
T-2	1.4 ± 1.1	0.7 ± 0.4	0.1 ± 0.0
FECES			
TOL	4.9 ± 1.9	5.0 ± 2.5	3.1 ± 0.5
deTOL	4.8 ± 1.0	6.0 ± 4.9	4.6 ± 1.1
M3	0.5 ± 0.3	1.7 ± 1.1	2.3 ± 1.8
M5	1.4 ± 0.4	0.7 ± 0.1	0.8 ± 0.3
M6	2.4 ± 1.6	4.1 ± 2.5	0.5 ± 0.2
M7	0.1 ± 0.1	1.2 ± 0.4	0.2 ± 0.03
3'OH HT-2	15.7 ± 4.8	19.9 ± 7.4	15.1 ± 4.4
M9	12.5 ± 0.6	17.9 ± 11.8	11.7 ± 0.2
3'OH T-2			
Triol	1.3 ± 0.4	4.1 ± 1.9	1.5 ± 0.2
M12	1.5 ± 0.3	1.7 ± 0.6	1.7 ± 0.5
deTriol	1.1 ± 0.2	1.2 ± 0.3	0.2 ± 0.2
HT-2	35.3 ± 3.2	30.7 ± 14.5	37.6 ± 3.0
deHT-2	18.5 ± 7.7	6.2 ± 6.2	13.8 ± 1.0
T-2		0.7 ± 0.7	6.2 ± 4.5

Table II.15b Metabolic profiles expressed as a percent of the extracted radioactivity in the excreta of rabbits 24 hours after administration of tritium-labeled T-2 toxin.

Metabolite	0.6 mg/kg I.V.	0.15 mg/kg Dermal	0.6 mg/kg Dermal
URINE			
TOL	38.2 ± 3.0	28.9 ± 4.4	31.0 ± 2.7
deTOL	2.2 ± 1.2	10.2 ± 3.5	1.4 ± 0.8
M3	4.8 ± 0.7	6.0 ± 0.4	6.4 ± 0.4
M5	2.8 ± 1.2	2.1 ± 1.3	2.8 ± 0.6
M6	0.7 ± 0.1	1.1 ± 0.3	1.0 ± 0.1
M7	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
3'OH HT-2	40.5 ± 3.4	37.6 ± 3.1	41.9 ± 2.1
M9	3.2 ± 0.7	6.9 ± 1.9	3.5 ± 1.3
3'OH T-2	1.0 ± 0.6	0.8 ± 0.4	1.0 ± 0.4
Triol	0.3 ± 0.1	0.5 ± 0.2	1.2 ± 0.7
M12	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.2
deTriol	0.1 ± 0.02	0.1 ± 0.1	0.2 ± 0.1
HT-2	4.9 ± 0.8	5.0 ± 2.3	6.5 ± 0.6
deHT-2	0.4 ± 0.3	0.04 ± 0.03	
T-2	0.1 ± 0.01	1.7 ± 0.5	1.9 ± 0.5
FECES			
TOL	1.9 ± 0.5	1.8 ± 0.4	6.9 ± 2.6
deTOL	0.8 ± 0.2	11.0 ± 0.7	11.2 ± 1.7
M3	0.8 ± 0.7	4.1 ± 2.1	2.2 ± 0.7
M5	1.6 ± 1.0	3.5 ± 2.7	2.9 ± 1.2
M6	0.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.2
M7	0.3 ± 0.2	1.0 ± 0.1	0.8 ± 0.4
3'OH HT-2	11.8 ± 1.8	8.7 ± 0.3	23.3 ± 10.3
M9	2.6 ± 0.6	30.5 ± 4.1	14.7 ± 5.5
3'OH T-2			
Triol	1.0 ± 0.2	2.4 ± 0.1	1.3 ± 0.2
M12	1.3 ± 0.2	1.2 ± 0.6	1.3 ± 0.1
deTriol	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
HT-2	76.1 ± 1.6	17.2 ± 1.9	16.3 ± 2.2
deHT-2		17.1 ± 2.6	4.1 ± 4.1
T-2	0.3 ± 0.2		14.6 ± 2.2

Table II.16a Metabolic profiles expressed as a percent of the extracted radioactivity in the excreta of rabbits 24 to 48 hours after administration of tritium-labeled T-2 toxin.

Metabolite	Route and Dose: 0.15 mg/kg Oral	0.6 mg/kg Oral	0.15 mg/kg I.V.
URINE			
TOL	15.1 ± 3.8	24.4 ± 7.3	29.4 ± 3.9
deTOL	28.7 ± 7.4	12.7 ± 10.8	19.8 ± 3.2
M3	4.2 ± 1.2	3.0 ± 1.3	5.9 ± 0.6
M5	1.1 ± 0.3	1.2 ± 0.3	0.8 ± 0.1
M6	1.8 ± 0.2	2.2 ± 0.2	1.3 ± 0.2
M7	0.4 ± 0.1	0.4 ± 0.03	0.4 ± 0.1
3'OH HT-2	28.3 ± 6.3	36.4 ± 8.8	20.9 ± 2.0
M9	19.7 ± 1.5	12.2 ± 6.8	18.2 ± 3.9
3'OH T-2			
Triol	0.5 ± 0.3	1.1 ± 0.4	1.2 ± 0.3
M12	0.4 ± 0.1	0.7 ± 0.3	0.2 ± 0.1
deTriol	0.04 ± 0.03	0.1 ± 0.1	0.1 ± 0.1
HT-2	1.3 ± 0.6	2.2 ± 0.8	1.5 ± 0.1
deHT-2	0.1 ± 0.1	0.1 ± 0.04	0.1 ± 0.1
T-2	1.7 ± 0.8	2.1 ± 1.4	
FECES			
TOL	3.7 ± 1.0	7.9 ± 5.7	0.5 ± 0.1
deTOL	28.8 ± 3.9	17.1 ± 8.5	33.8 ± 6.1
M3	0.9 ± 0.1	0.4 ± 0.3	4.8 ± 4.1
M5	1.7 ± 0.7	1.2 ± 0.5	0.6 ± 0.1
M6	1.2 ± 0.1	2.8 ± 1.5	1.1 ± 0.2
M7	1.1 ± 0.2	1.6 ± 0.8	2.7 ± 1.5
3'OH HT-2	11.6 ± 3.1	24.5 ± 13.8	6.0 ± 0.9
M9	41.6 ± 3.1	26.2 ± 15.7	44.4 ± 1.0
3'OH T-2			
Triol	2.5 ± 0.3	3.4 ± 1.1	2.3 ± 0.1
M12	0.0	0.4 ± 0.3	0.2 ± 0.2
deTriol	0.5 ± 0.3	0.7 ± 0.1	0.4 ± 0.2
HT-2	3.0 ± 1.6	8.0 ± 5.7	0.5 ± 0.2
deHT-2	2.9 ± 0.8	6.2 ± 1.7	2.0 ± 0.7
T-2	0.8 ± 0.3		0.7 ± 0.3

Table II.16b Metabolic profiles expressed as a percent of the extracted radioactivity in the excreta of rabbits 24 to 48 hours after administration of tritium-labeled T-2 toxin.

Metabolite	Route and Dose: 0.6 mg/kg I.V.	0.15 mg/kg Dermal	0.6 mg/kg Dermal
URINE			
TOL	30.9 ± 6.7	17.7 ± 2.2	36.0 ± 6.3
deTOL	17.3 ± 9.1	21.2 ± 5.7	5.8 ± 5.1
M3	3.8 ± 1.2	5.1 ± 1.0	7.5 ± 0.3
M5	1.7 ± 0.8	1.0 ± 0.1	2.2 ± 0.4
M6	1.2 ± 0.3	1.1 ± 0.1	1.7 ± 0.3
M7	0.2 ± 0.01	0.5 ± 0.1	0.1 ± 0.02
3'OH HT-2	27.3 ± 7.8	23.1 ± 2.0	31.6 ± 4.3
M9	15.2 ± 7.6	17.5 ± 3.1	8.2 ± 3.5
3'OH T-2 Triol	0.7 ± 0.3	1.2 ± 0.5	0.8 ± 0.2
M12	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
deTriol	0.04 ± 0.03	0.1 ± 0.01	0.1 ± 0.02
HT-2	0.8 ± 0.2	1.8 ± 0.5	3.0 ± 0.6
deHT-2	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
T-2		2.9 ± 0.5	2.2 ± 0.2
FECES			
TOL	12.3 ± 10.0	0.6 ± 0.1	10.3 ± 7.6
deTOL	20.5 ± 16.5	31.0 ± 1.1	17.5 ± 9.8
M3	1.1 ± 0.1	0.0 ---	2.9 ± 0.9
M5	2.9 ± 2.1	0.9 ± 0.2	2.1 ± 0.8
M6	2.6 ± 1.4	1.1 ± 0.1	1.4 ± 0.6
M7	1.5 ± 0.2	2.7 ± 1.1	0.9 ± 0.3
3'OH HT-2	29.2 ± 18.2	6.3 ± 0.7	26.6 ± 14.1
M9	24.4 ± 16.5	46.6 ± 1.2	24.4 ± 9.8
3'OH T-2 Triol	1.0 ± 0.5	2.8 ± 0.1	1.2 ± 0.4
M12	0.0 ---	0.7 ± 0.3	0.8 ± 0.4
deTriol	0.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.4
HT-2	2.7 ± 2.1	1.7 ± 0.8	4.3 ± 1.4
deHT-2	0.7 ± 0.4	3.3 ± 1.2	2.3 ± 1.3
T-2		1.7 ± 0.6	3.7 ± 1.3

Table II.17 Metabolic profiles expressd as a percent of the extracted radioactivity in the excreta of rabbits 48 to 72 hours after administration of tritium-labeled T-2 toxin.

Metabolite	Route and Dose: 0.15 mg/kg Oral	0.6 mg/kg Oral	0.15 mg/kg I.V.
URINE			
TOL	9.7 ± 1.0	23.7 ± 9.8	11.1 ± 3.1
deTOL	35.2 ± 4.2	18.4 ± 14.2	38.4 ± 5.5
M3	2.8 ± 0.6	3.6 ± 1.1	2.5 ± 0.4
M5	1.1 ± 0.4	1.2 ± 0.3	0.8 ± 0.2
M6	1.5 ± 0.3	3.5 ± 0.5	1.0 ± 0.2
M7	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
3'OH HT-2	17.5 ± 2.2	30.6 ± 7.9	16.1 ± 0.8
M9	24.8 ± 3.7	12.7 ± 7.9	27.0 ± 1.7
3'OH T-2 Triol	0.8 ± 0.2	1.1 ± 0.5	1.2 ± 0.4
M12	0.3 ± 0.1	0.7 ± 0.3	0.01 ± 0.01
deTriol	0.1 ± 0.03	0.2 ± 0.1	0.1 ± 0.03
HT-2	1.0 ± 0.3	1.6 ± 0.7	0.7 ± 0.4
deHT-2	0.1 ± 0.05	0.2 ± 0.1	0.1 ± 0.1
T-2	4.1 ± 3.2	1.6 ± 1.0	0.2 ± 0.1
FECES			
TOL	2.8 ± 1.2	18.2 ± 8.9	1.0 ± 0.2
deTOL	40.3 ± 2.0	15.2 ± 10.7	39.8 ± 4.9
M3	1.1 ± 0.3	2.9 ± 1.3	31.2 ± 0.4
M5	1.3 ± 0.6	1.4 ± 0.4	4.7 ± 3.9
M6	0.7 ± 0.2	8.5 ± 3.9	0.8 ± 0.2
M7	1.8 ± 0.3	2.2 ± 1.0	3.0 ± 1.7
3'OH HT-2	9.4 ± 1.7	28.8 ± 7.7	11.4 ± 1.0
M9	38.2 ± 2.2	16.7 ± 10.3	35.1 ± 1.3
3'OH T-2	ND	ND	ND
Triol	2.4 ± 0.8	2.6 ± 1.1	1.4 ± 0.2
M12	0.4 ± 0.4	0.3 ± 0.3	0.3 ± 0.2
deTriol	0.4 ± 0.2	0.6 ± 0.3	0.3 ± 0.2
HT-2	0.4 ± 0.1	2.6 ± 1.6	0.3 ± 0.1
deHT-2	0.3 ± 0.1	0.6 ± 0.3	2.2 ± 1.9
T-2	0.1 ± 0.02	0.5 ± 0.2	ND

Table II.18a Combined metabolic profiles over days 1, 2 and 3 expressed as a percent of the administered dose in rabbits administered tritium-labeled T-2 toxin.

Metabolite	Route and Dose: 0.15 mg/kg Oral	0.6 mg/kg Oral	0.15 mg/kg I.V.
URINE			
TOL	3.5 ± 0.8	6.6 ± 0.8	8.3 ± 0.4
deTOL	2.5 ± 0.2	3.2 ± 2.5	2.2 ± 0.2
M3	0.9 ± 0.3	1.2 ± 0.2	1.6 ± 0.2
M5	0.2 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
M6	0.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
M7	0.1 ± 0.01	0.2 ± 0.1	0.1 ± 0.01
3'OH HT-2	5.3 ± 1.2	10.4 ± 1.6	8.4 ± 0.6
M9	2.0 ± 0.4	2.7 ± 1.8	2.2 ± 0.3
3'OH T-2	0.01 ± 0.02	0.1 ± 0.04	0.1 ± 0.02
Triol	0.1 ± 0.02	0.8 ± 0.5	0.2 ± 0.1
M12	0.1 ± 0.03	0.2 ± 0.1	0.1 ± 0.02
deTriol	0.3 ± 0.01	0.1 ± 0.02	0.02 ± 0.003
HT-2	0.3 ± 0.2	1.4 ± 0.4	1.4 ± 0.2
deHT-2		0.03 ± 0.02	0.01 ± 0.01
T-2	1.4 ± 1.3	0.3 ± 0.1	0.01 ± 0.01
Total	16.5 ± 3.4	28.7 ± 7.08	25.3 ± 1.1
FECES			
TOL	0.4 ± 0.1	0.7 ± 0.5	0.1 ± 0.01
deTOL	2.3 ± 0.8	2.1 ± 1.5	1.7 ± 0.1
M3	0.1 ± 0.01	0.1 ± 0.0	30.2 ± 0.2
M5	0.1 ± 0.02	0.1 ± 0.0	20.1 ± 0.1
M6	0.1 ± 0.03	0.4 ± 0.2	0.1 ± 0.01
M7	0.1 ± 0.03	0.2 ± 0.1	0.2 ± 0.04
3'OH HT-2	1.2 ± 2.1	2.1 ± 0.6	0.5 ± 0.1
M9	3.0 ± 0.8	3.1 ± 2.2	2.1 ± 0.3
3'OH T-2			
Triol	0.2 ± 0.02	0.3 ± 0.2	0.1 ± 0.01
M12	0.1 ± 0.02	0.03 ± 0.02	0.03 ± 0.01
deTriol	0.1 ± 0.02	0.1 ± 0.0	0.5 ± 0.1
HT-2	1.2 ± 0.4	0.5 ± 0.3	0.3 ± 0.1
deHT-2	0.4 ± 0.1	0.6 ± 0.3	0.1 ± 0.1
T-2	0.1 ± 0.02	0.02 ± 0.2	
Total	9.2 ± 2.6	10.4 ± 2.7	5.9 ± 0.7

Table II.18b Combined metabolic profiles over days 1, 2 and 3 expressed as a percent of the administered dose in rabbits administered tritium-labeled T-2 toxin.[a]

Metabolite	Route and Dose: 0.6 mg/kg I.V.	0.15 mg/kg Dermal	0.6 mg/kg Dermal
URINE			
TOL	8.6 ± 1.7	3.7 ± 0.3	3.7 ± 0.2
deTOL	1.3 ± 0.7	2.5 ± 0.6	0.5 ± 0.3
M3	1.1 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
M5	0.7 ± 0.4	0.3 ± 0.2	0.3 ± 0.1
M6	0.2 ± 0.1	0.2 ± 0.04	0.1 ± 0.04
M7	0.1 ± 0.02	0.1 ± 0.02	0.03 ± 0.01
3'OH HT-2	9.3 ± 2.9	4.7 ± 0.9	4.0 ± 1.1
M9	1.4 ± 0.4	1.8 ± 0.3	0.7 ± 0.3
3'OH T-2	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Triol	0.1 ± 0.01	0.1 ± 0.04	0.2 ± 0.1
M12	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.03
deTriol	0.02 ± 0.01	0.02 ± 0.003	0.02 ± 0.01
HT-2	0.9 ± 0.3	0.4 ± 0.1	0.5 ± 0.2
deHT-2	0.1 ± 0.1	0.02 ± 0.003	0.01 ± 0.003
T-2	0.1 ± 0.1	0.3 ± 0.04	0.3 ± 0.02
Total	24.1 ± 4.5	15.0 ± 2.2	11.3 ± 1.95
FECES			
TOL	1.3 ± 1.0	0.1 ± 0.03	0.4 ± 0.3
deTOL	0.8 ± 0.6	1.5 ± 0.6	0.4 ± 0.1
M3	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.01
M5	0.5 ± 0.1	0.1 ± 0.01	0.1 ± 0.1
M6	0.3 ± 0.2	0.1 ± 0.02	0.1 ± 0.02
M7	0.1 ± 0.02	0.2 ± 0.1	0.03 ± 0.01
3'OH HT-2	2.9 ± 2.0	0.5 ± 0.2	1.0 ± 0.7
M9	1.0 ± 0.4	2.3 ± 0.8	0.4 ± 0.9
3'OH T-2	0.1 ± 0.1	ND	0.01 ± 0.003
Triol	0.1 ± 0.01	0.2 ± 0.1	0.03 ± 0.0
M12	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.003
deTriol	0.03 ± 0.01	0.03 ± 0.003	0.01 ± 0.00
HT-2	1.2 ± 0.4	0.4 ± 0.1	0.2 ± 0.03
deHT-2	0.03 ± 0.01	0.5 ± 0.2	0.04 ± 0.03
T-2	0.01 ± 0.001	0.1 ± 0.02	0.2 ± 0.01
Total	8.3 ± 2.8	5.9 ± 1.8	2.9 ± 1.0

[a] x ± SE (n = 3)

Figure II.26 Excretion of radioactivity after oral administration of T-2 toxin at 0.15 mg/kg to rabbits in terms of cumulative excretion and daily excretion in urine and feces.

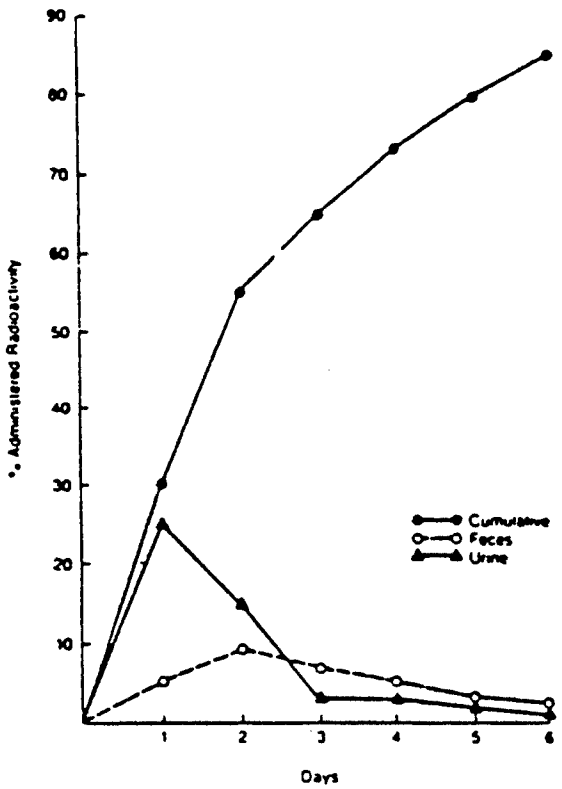


Figure II.27 Excretion of radioactivity after oral administration of T-2 toxin at 0.60 mg/kg to rabbits in terms of cumulative excretion and daily excretion in urine and feces.

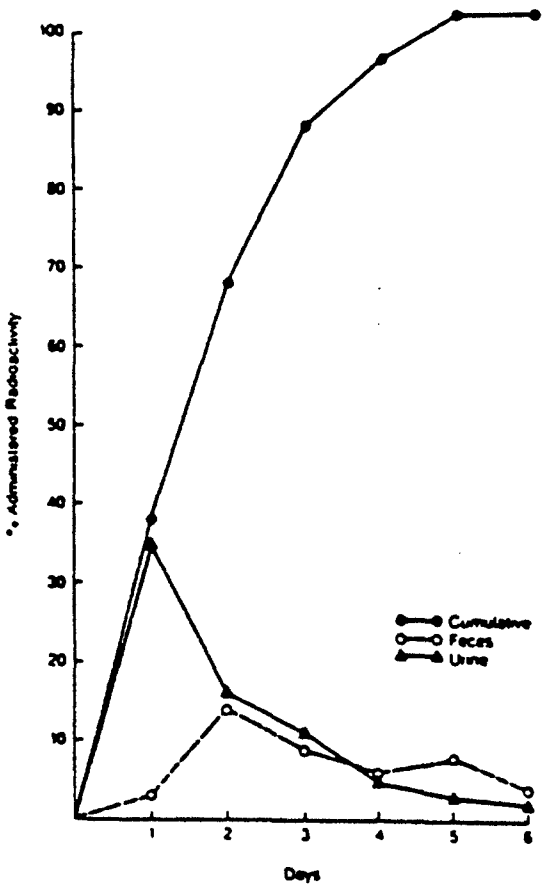


Figure II.28 Excretion of radioactivity after i.v. administration of T-2 toxin at 0.15 mg/kg to rabbits in terms of cumulative excretion in urine and feces.

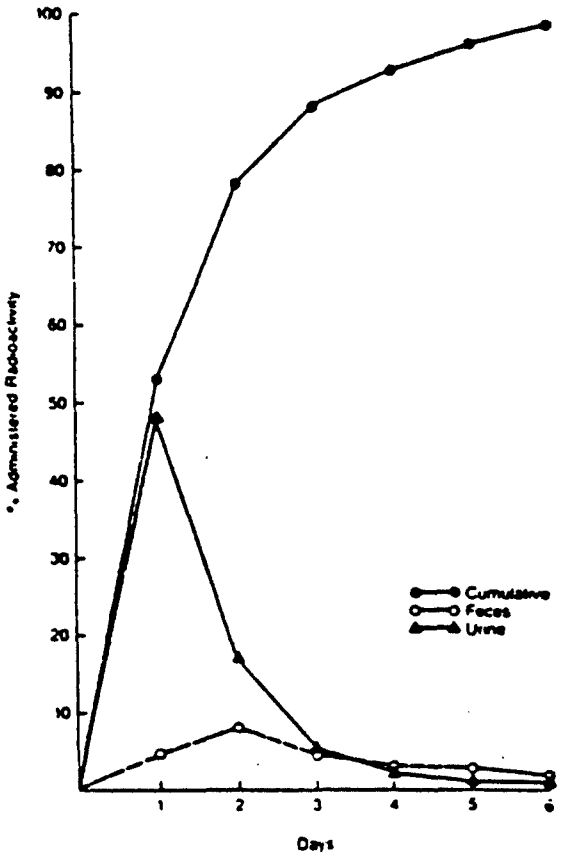


Figure II.29 Cummulative elimination of radioactivity in urine and feces following dermal administration of T-2 toxin to rabbits at 0.15 mg/kg body weight.

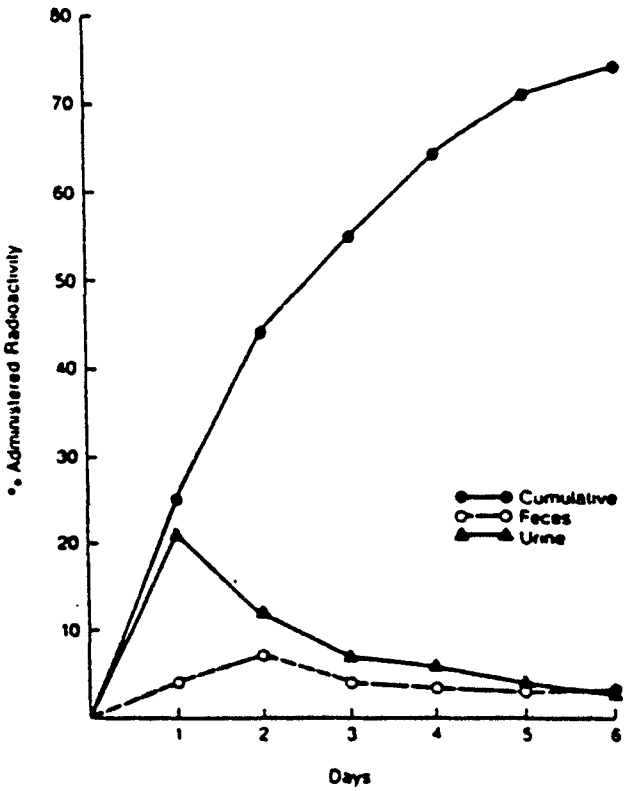


Figure II.30 Cummulative elimination of radioactivity in urine and feces following dermal administration of T-2 toxin to rabbits at 0.60 mg/kg body weight.

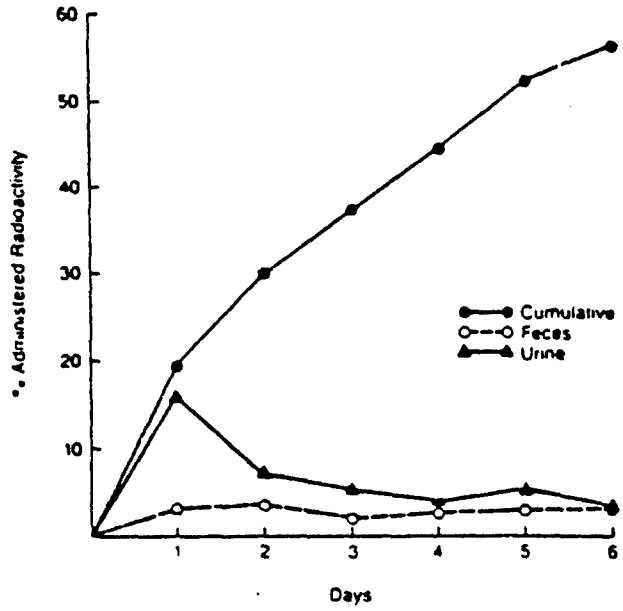
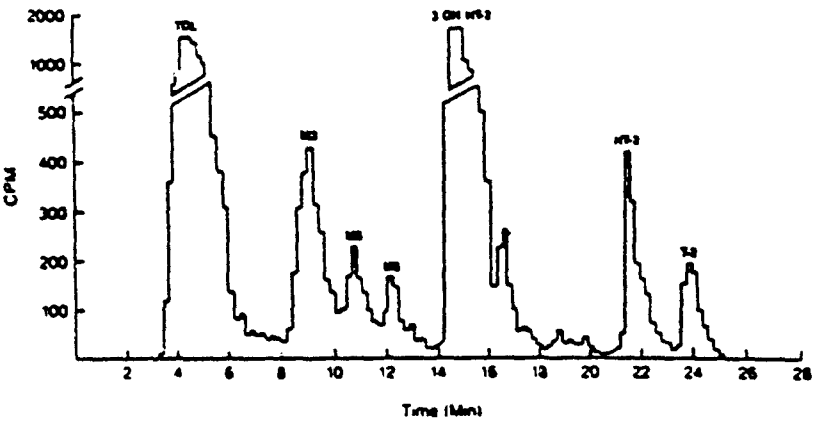


Figure II.31 HPLC radiochromatogram of rabbit urine (A) and rat feces (B).

(A)



(B)

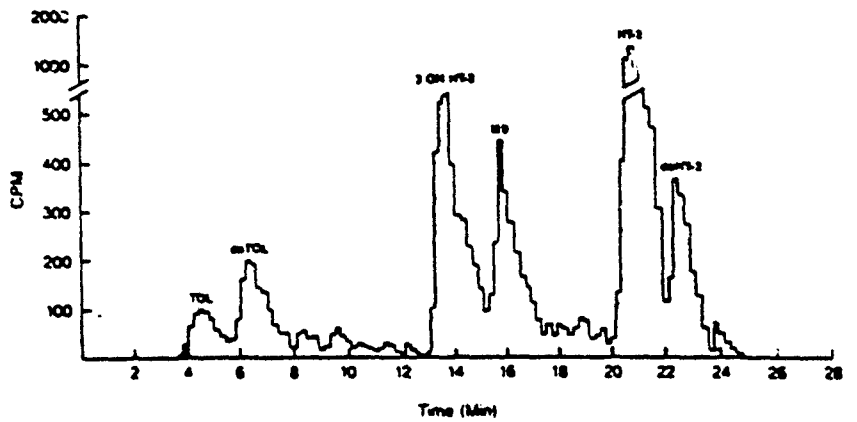


Figure II.32 Effect of time on selected metabolites in rabbit urine expressed as percent of extracted radicactivity (perofext). (* denotes $p < 0.05$.)

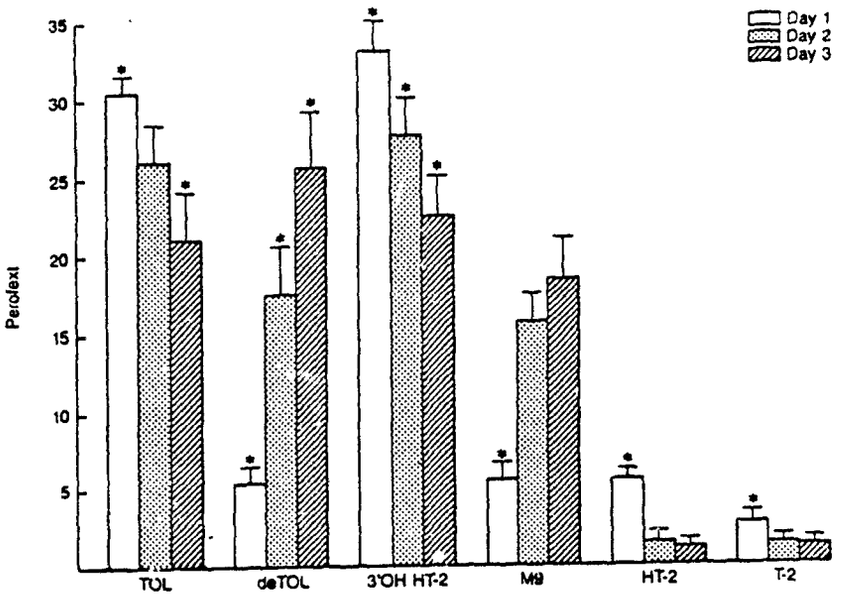


Figure II.33 Effect of dose on selected metabolites in rabbit urine expressed as percent of extracted radioactivity (percext).

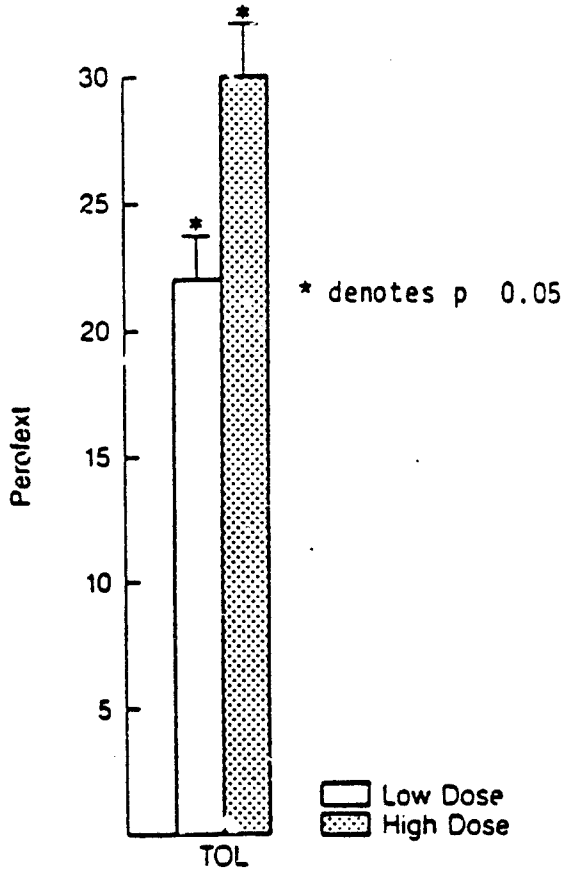


Figure II.34 Effect of route on selected metabolites in rabbit urine expressed as percent of extracted radioactivity.

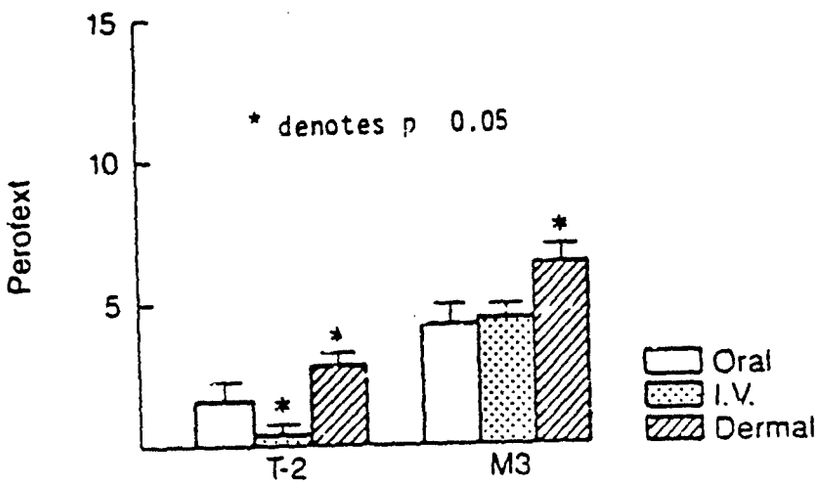


Figure II.35 Effect of dose on selected metabolites in rabbit feces expressed as percent of extracted radioactivity (perofext).

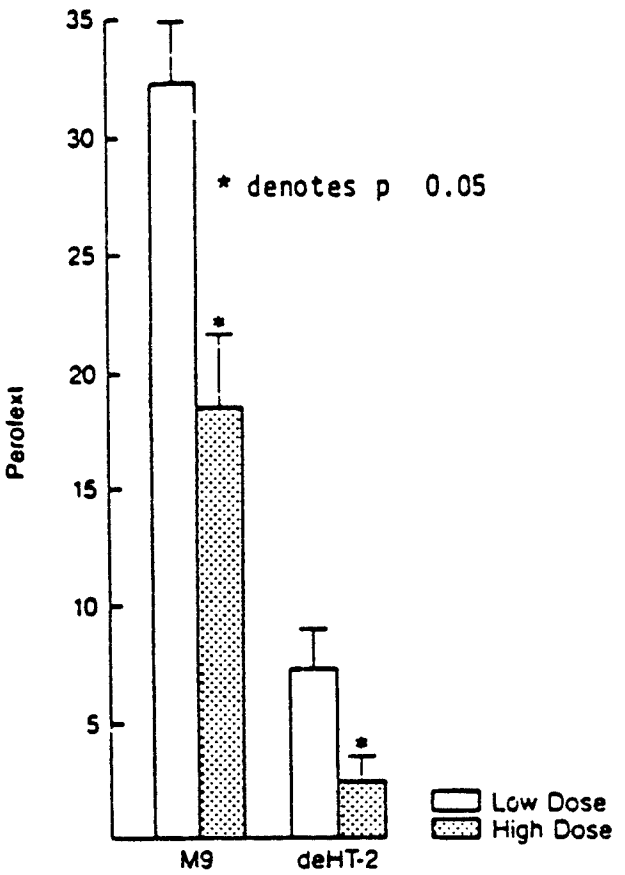


Figure II.36 Effect of route on selected metabolites in rabbit feces expressed as percent of extracted radioactivity (perofext).

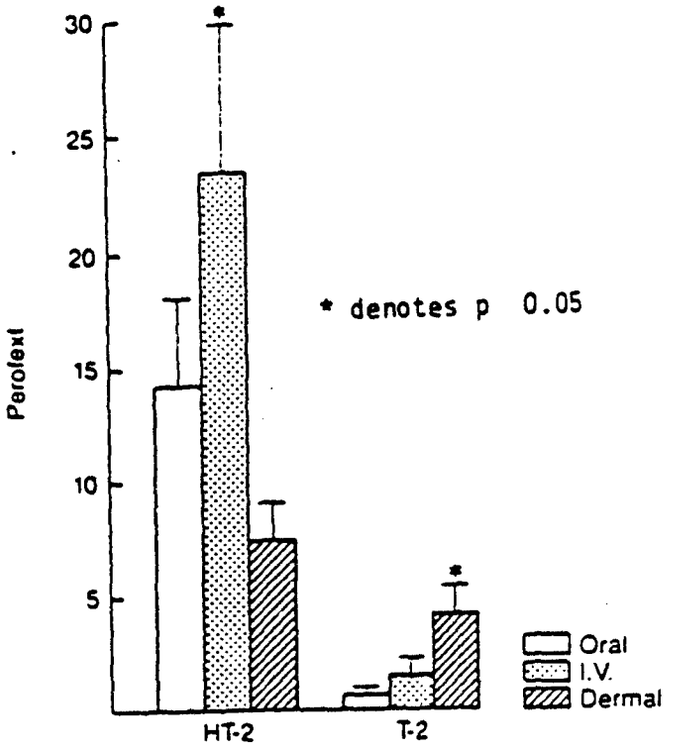
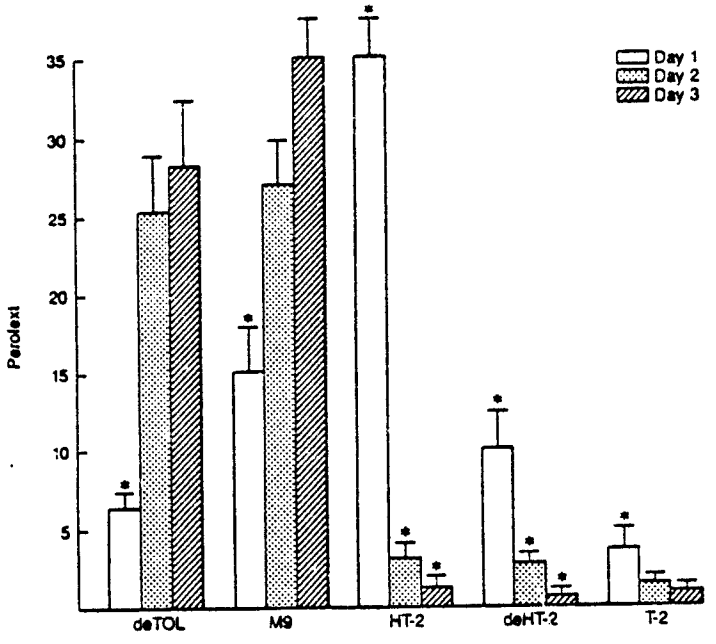


Figure II.37 Effect of time on selected metabolites in rabbit feces expressed as percent of extracted radioactivity (perofext). (* denotes $p < 0.05$.)



C. Analytical Methodology

1. Gas chromatographic screening method for T-2 toxin, diacetoxyscirpenol, deoxynivalenol, and related trichothecenes in feeds

by

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Abstract

A gas chromatographic method for screening trichothecene mycotoxins in feeds is described. Feed is extracted with acetonitrile-water, and the toxins purified with charcoal/alumina, florisil, and silica mini-columns. Deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), T-2 toxin, and their fungal metabolites are hydrolyzed to their corresponding parent alcohols (DON, NIV, scirpentriol, or T-2 tetraol) by alkaline hydrolysis. After derivatization to their pentafluoropropionyl analogs, the toxins are quantitated by gas chromatography with electron capture detection. Confirmation and increased sensitivity can be achieved by negative chemical ionization mass spectrometry with no additional sample workup. Recoveries of DAS, DON, and T-2 toxin averaged, respectively, 80%, 65%, and 85% in corn; 84%, 65%, and 88% in soybeans; and 70%, 57%, and 96% in mixed feeds at concentrations ranging from 0.1 to 2.0 ppm. Recovery of the polar trichothecene nivalenol averaged 86% in corn. A detection limit of 0.02 ppm in corn, soybeans, and mixed feeds and 0.05 ppm in silages is estimated.

Introduction

Deoxynivalenol (DON, vomitoxin), nivalenol (NIV), diacetoxyscirpenol (DAS), and T-2 belong to a group of related secondary fungal metabolites called trichothecenes. These toxins are four of the more important members of this class of mycotoxins detected in agricultural products (Pathre and Mirocha, 1979; Bamburg and Strong, 1971; Ghosal et al., 1978; Mirocha et al., 1976; Pathre and Mirocha, 1977; Smalley and Strong, 1974). Trichothecenes are produced by several genera of fungi and particularly species of the genus Fusarium (Mirocha et al., 1976; Doyle and Bradner, 1980; Kosuri et al., 1970; Scott et al., 1980; Ueno et al., 1972; Hsu et al., 1972; Vesonder et al., 1973; Morooka et al., 1972). Consumption of trichothecene contaminated grains by livestock can result in a variety of adverse health effects including: emesis, diarrhea, lethargy, reduced weight gain, embryotoxicity, decreased immune response, and death (Pathre and Mirocha, 1979; Bamburg and Strong, 1971; Côté et al., 1984; Forsyth et al., 1977; Sato and Ueno, 1977; Obara et al., 1984; Mirocha et al., 1977; Corrier and Ziprin, 1986).

Species of Fusarium have the capacity to simultaneously produce several related trichothecenes. To date, toxicological research has focused primarily on the effects of individual toxins. The combined effect of these toxins on livestock is not known. Presently, over 60 different trichothecenes have been identified. Simultaneous analysis for all trichothecenes is difficult due to the large variation in chemical substituents on the basic trichothecene ring. This substituent variation results in considerable diversity in compound polarity. This paper presents a gas chromatographic method for screening DON, DAS, T-2 toxin,

and related trichothecenes in grains with a single analysis. Conditions for mass spectrometric confirmation are also described.

Method

Apparatus

a. Gas Chromatograph. Hewlett Packard 5840A, equipped with a ^{63}Ni electron capture detector and 7672A autosampler. DB-1701 Megabore capillary column, 15 m x 0.527 mm id and 1 μ film thickness (J & W Scientific, Folsom, CA).

b. Mass Spectrometer. Extranuclear (Extrel) Simulscan 300 Series quadrapole with a Perkin Elmer Sigma 2 gas chromatograph. Negative chemical ionization with methane.

c. Vacuum Manifold. Vac Elut manifold (Analytichem International, Harbor City, CA).

d. Mini-Columns. Seven mL polyethylene columns with paper frits, bottom closures, and reserviors (Iso Labs, Lincoln, NE).

e. Filter Paper Disks. Schleicher & Schuell, 1/2-inch diameter (American Scientific Products, MacGaw Park, IL).

f. Silica Cartridges. Prep Sep, 300 mg of packing (Fisher Scientific, Itasca, IL).

g. Rotary Evaporator. Buchi RE120 (Brinkman Instruments, Inc., Westbury, NY).

h. Evaporator. Meyer N-Evap (Organomation Associates, Inc., South Berlin, MA).

Reagents

a. All glass distilled solvents were used (EM Science, Cherry Hill, NJ) except for ACS grade acetonitrile used for initial feed extraction (Fisher Scientific, Itasca, IL).

b. Charcoal/Alumina Columns. Activiated charcoal, Darco G-60 (MCB Manufacturing Chemists, Inc., Cincinnati OH). Neutral alumina, Brockman Activity 1, 80 to 200 mesh (Fisher Scientific, Itasca, IL). Celite 545, (J. T. Baker Chemical Co., Phillipsburg, NJ). Add 2 g of charcoal-alumina-celite (1 + 1 + 1, w/w/w) to a polyethylene mini-column. Gently tap the column to settle the absorbant. Place a filter paper disk on top of the packing material. These columns may be prepared in advance and stored in a sealed container.

c. Florisil. Sixty to 100 mesh (Fisher Scientific, Itasca, IL).

d. 4-dimethylaminopyridine (DMAP). Sigma Chemical Co., St. Louis, MO.

e. Pentafluoropropionic Acid Anhydride (PFPA). Pierce Chemical Co., Rockford, IL.

f. Deoxynivalenol. Myco Lab Co., Chesterfield, MO.

g. Nivalenol. Wako Chemical USA, Inc., Dallas, TX.

h. T-2 tetraol and HT-2 were prepared by the alkaline hydrolysis of T-2; scirpentriol and 15-monoacetoxyscirpenol (MAS) were prepared by the alkaline hydrolysis of DAS (20). T-2 toxin and DAS were isolated and purified from fungal cultures of Fusarium sporotrichioides grown in our laboratory.

i. Internal Standard. Deepoxy T-2 tetraol (de TOL) at 5 ng/uL in absolute ethanol. Deepoxy T-2 tetraol was prepared by incubating T-2 with rumen microorganisms under an anaerobic environment (21) followed by alkaline hydrolysis of the resulting deepoxy metabolites.

Extraction

Place 50 g of ground feed and 100 mL of 90% acetonitrile in 250 mL Erlenmeyer flask and vigorously shake for 1 hour. Filter extract through Whatman #4 filter paper.

Charcoal/alumina/celite column

Using vacuum apparatus described by Romer (22), wet charcoal/alumina/celite column with 5 ml of acetonitrile-water (84 + 16). Discard wetting solvent. Add 4 ml feed extract to column, apply vacuum, and collect eluate in a 125-m; round-bottom flask. Elute toxins with 35 ml acetonitrile-water (84 + 16) and collect eluate until flow from column has stopped. Collect eluate in same 125-ml round-bottom flask containing initial eluate. Do not run the column to dryness at any time prior to final elution; otherwise, recoveries of toxins will decrease. Rinse neck of flask with 20 ml of acetonitrile. Concentrate solvent to dryness using a rotary evaporator, additional acetonitrile may be necessary to completely remove all traces of water.

Florisil Mini-Column

Using hexane, wet pack 0.5 g of Florisil in a polyethylene mini-column fitted with a bottom closure and reservior. Layer 5 mm of granular sodium sulfate above and below the packing. Drain any excess hexane to the top of the upper sodium sulfate layer then add 1 ml of hexane to the column. Dissolve the residue from the charcoal/alumina column in 1 ml of ethyl acetate-methanol (3 + 1) and transfer to the Florisil column. Rinse the flask with 2 x 1 ml of ethyl acetate and transfer to the column. Drain the transfer solvents and collect the eluate. Add 4 ml of ethyl acetate to the column and collect with the previous column eluate. Concentrate to dryness in a 50°C water bath with a stream of dry nitrogen.

Hydrolysis

Add 125 µl of 0.15 N NaOH in methanol-water (95 + 5) to the concentrated residue. Cap, vortex-mix, and incubate 35 minutes at room temperature. After the incubation period, add 60 µl of 0.325 N acetic acid in toluene and vortex-mix.

Silica Cartridge

Condition a silica cartaridge with 3 ml of methylene chloride-acetone (1 + 4) followed by 3 ml of hexane. Add 3 ml of methylene chloride-hexane

(1 + 3) to the neutralized hydrolysis solution, vortex-mix, and transfer to cartridge. Apply vacuum and discard the eluate. Add 2 ml of methylene chloride-hexane (2 + 1) to hydrolysis test tube, vortex, and transfer to cartridge. Apply vacuum and discard eluate. Elute the toxins with 7 ml of methylene chloride-acetone (1 + 4); use first 2 ml to aid final transfer of residues. Add 100 µl of the de TOL internal standard solution to the eluate. Concentrate to dryness in a 50°C water bath with stream of dry nitrogen.

Derivatization

Add 0.5 ml of toluene-acetonitrile (90 + 10) containing 2 mg/ml DMAP to the residue. Add 50 µl of PFPA, cap and heat for 20 minutes at 60°C. Cool to room temperature. Add 1 ml of 5% sodium bicarbonate and vortex for 15 to 20 seconds. Add 0.5 ml of iso-octane and vortex until the top layer is clear. Carefully remove and discard the bottom layer. Add 1 ml of water and vortex for 15 to 20 seconds. Centrifuge at 1,000 rpm for 2 to 3 minutes. Transfer 50 µl of the top layer to a screw cap test tube or autosampler vial. Add 0.95 ml of iso-octane and vortex.

Dilution Scheme For Mass Spectroscopy

Transfer 200 µl of the organic layer to a small vial. Add 200 µl of iso-octane and vortex. Inject 1 µl into the gas chromataograph/mass spectrometer.

Gas Chromatography

Inject 1 µl of the derivatized sample using the following conditions: oven temperature of 195°C; injector temperature of 250°C; detector temperature of 300°C; helium carrier gas at a flow rate of 7 cc/minute; argon/methane (95 + 5) detector add-on gas giving a total flow of 42 ml/minute.

Results and Discussion

The structures of T-2 toxin, DAS, DON, NIV, and their fungal metabolites are shown in Figure II.38. In the method described, T-2 toxin and its metabolites are hydrolyzed to T-2 tetraol (TOL), DAS, and its metabolites are hydrolyzed to scirpentriol (STR), 3-acetyldeoxynivalenol is hydrolyzed to deoxynivalenol (DON), and fusanenon-X is hydrolyzed to nivalenol (NIV), upon treatment with 0.15 N NaOH. T-2 tetraol, scirpentriol, deoxynivalenol, and nivalenol do not contain ester side chains and are referred to as the pa .t alcohols for each of their respective trichothecene groups. All of the esterified trichothecenes listed in Figure II.38 were converted to their respective parent alcohols under the hydrolysis conditions described. Deoxynivalenol and nivalenol, lacking ester side groups, are not hydrolyzed under these conditions.

The rate of ester hydrolysis and degradation of the trichothecene skeleton varies for each respective group of toxins. The isovaleryl ester at the C-8 position of T-2 is the most resistant to cleavage. Trichothecenes with a C-8 ketone, such as DON and NIV, are much more susceptible to degradation under alkaline conditions (Rood et al., 1986; Young et al., 1986). The hydrolysis conditions were selected to minimize the degradation of DON and NIV and to maximize the conversion of T-2 to TOL and DAS to STR. The rate of hydrolysis to the corresponding parent

alcohols varied due to the sample matrix (data not shown). Accordingly, hydrolysis condtions were selected to obtain the highest overall recovery of the toxins from a variety of feed matrices.

Trichothecenes with ester side chains are converted to their corresponding parent alcohols under the hydrolysis conditions described. This provides a greater analytical sensitivity in detecting trichothecenes for two reasons. First, ester hydrolysis leads to a cummulative effect since all of the metabolites within a given group are hydrolyzed to a single compound, the parent alcohol. Second, the pentafluoropropionyl (PFP) derivatives of the parent alcohols have substantially greater response factors by electron capture detection and negative chemical ionization mass spectrometry than the esterified trichothecenes, compared with the PFP derivatives of the esterified trichothecenes.

Detector responses for the derivatized toxins ranged from 5 to 8 pg/cm peak height by GLC/ECD. Ten pg of derivatized parent alcohol could be detected by negative chemical ionization mass spectrometry. Based on this criteria, the detection limit is estimated to be 50 ng/g for silages and 20 ng/g for corn, wheat, soybeans, and mixed feeds. Chromatograms for a variety of feeds naturally contaminated with trichothecenes are shown in Figure II.39.

For analysis below a concentration of 0.2 ppm, a different dilution scheme was required for the derivatized extract (toluene-acetonitrile/PFP) than for extracts containing higher levels of toxin. Dilution of the toluene-acetonitrile with iso-octane after the first vortex-mixing (with 5% bicarbonate) decreased the amount of polar contaminants remaining remaining in the organic layer. This resulted in a decreased solvent front and reduced tailing as compared to non isooctane diluted samples. An added benefit was the gas chromatograph injection liner required less frequent cleaning.

Acetontrile-water was selected as the extraction solvent, since it has been reported to efficiently extract a varety of trichothecenes (Romer, 1986; Trenholm et al., 1985). In the present study, toxin recoveries were determined by adding appropriate quantities of purifed compounds to the feed extracts. The resulting data is shown in Table II.13. Improved precision was achieved by adding an internal standard to the samples prior to derivatization, after the hydrolysis. Deepoxy T-2 tetraol was selected as the internal standard since it is nearly identical in structure to T-2 tetraol, and it has not been reported as a naturally occurring contaminant in feeds or as a fungal product in cultures.

To assess the recovery of polar trichothecenes by this method, experiments were performed where HT-2, TOL, NIV, and 15-monoacetoxyscirpenol (MAS) were added to feed extracts The recoveries of these compounds is shown in Table II.14.

Positive samples can be confirmed by gas chromatographic/mass spectrometric with no additional sample workup. The PFP derivatives have previously been recommended for mass spectrometric analysis due to their mass range and excellent sensitivity in the negative chemical ionization mode (Krishnamurthy and Sarver, 1986). The molecular ion and M-20 ions are the predominant fragments observed in the spectra (Figure II.40).

Variation amoung different lots of adsorbents has been reported (Majors, 1986; Rood et al., 1988), and we have noted varying adsorvent activity, not related to simple hydration, with different lots of Florisil from the same manufacturer. In particular, this affected the recovery of polar trichothecenes such as TOL and NIV; however, recoveries of nonpolar trichothecenes such as T-2 toxin and DAS were not adversely affected. To obtain TOL recoveries greater than 75%, one lot of Florisil required 7 ml of ethyl acetate-acetone (4 + 1), whereas another lot required 7 ml of ethyl acetate. Calibration of each lot of Florisil with a polar and nonpolar trichothecene (i.e., T-2 toxin and TOL) is necassary to ensure reproducible recoveries of all toxins. The use of activated Florisil (suitable for pesticied analysis) is not recommended because its high activity requires polar solvent mixtures containing methanol for toxin elution. This results in minimal removal of impurities from the extracted samples and leasds to interfering peaks in the gas chromatogram.

The described method is simple, because several trichthecene families (DON, DAS, T-2 toxin, plus their fungal metabolites) can be detected in a single analysis. Samples that are positive for trichothecenes by this screening method can be easily confirmed by mass spectrometry. The method is currently being used to screen samples submitted to the Veterinary Medicine Diagnostic Laboratory at the University of Illinois.

Acknowledgements

The authors would like to thank Phil Sanders for obtaining the mass spectra and Carla Helaszek, Gui-rong Wang and Roseann McCartney for their technical assistance. This work was supported in part by Contract No. DAMD-17-85-C-5224 from the U.S. Army Medical Research and Development Command and the USDA North Central Regional Project NC-129.

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Table II.19 Recovery of T-2 toxin (T-2), diacetoxyscirpenol (DAS), and deoxynivalenol (DON) in corn, soybeans, and mixed feed.

	Amount Added (ppm)	Toxin[a]	Recovery %[b]
Corn	0.1	DAS	91% ± 7
	0.1	T-2	93% ± 10
	0.1	DON	69% ± 8
	1.0	DAS	69% ± 2
	1.0	T-2	78% ± 3
	2.0	DON	61% ± 2
Soybeans	0.1	DAS	84% ± 7
	0.1	T-2	89% ± 7
	0.1	DON	70% ± 7
	1.0	DAS	85% ± 5
	1.0	T-2	87% ± 7
	2.0	DON	61% ± 4
Mixed feeds[b]	0.1	DAS	67% ± 4
	0.1	T-2	114% ± 10
	0.1	DON	52% ± 12
	1.0	DAS	56% ± 5
	1.0	T-2	77% ± 7
	2.0	DON	63% ± 2

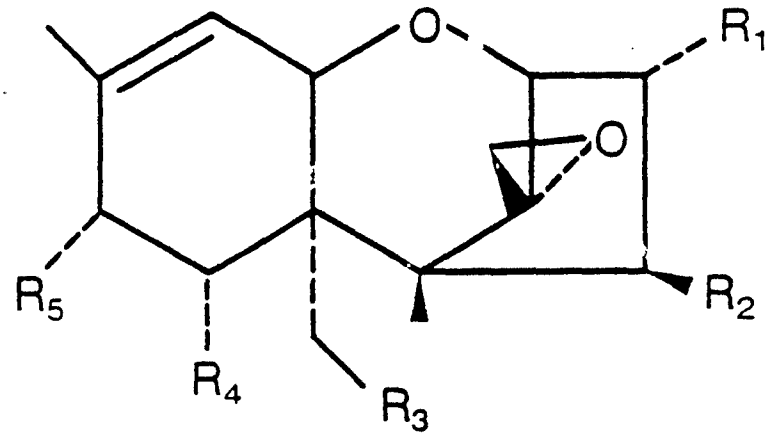
[a]T-2 toxin, DAS and DON were analyzed as their parent alcohols (T-2 tetraol, scirpentriol, and DON, respectively) after alkaline hydrolysis.
[b]Mean +/- standard error; n = 8.
[c]Mixed feed contained soybean meal, meat meal, ground corn, and milo.

Table II.20 Recovery of 15-monoacetoxyscirpenol (MAS), HT-2, nivalenol (NIV), and T-2 tetraol (TOL) from corn.

Amount Added (ppm)	Toxin[a]	Recovery[b]
0.25	MAS	97% ± 7
0.25	HT-2	97% ± 3
0.25	NIV	86% ± 10
0.25	TOL	56% ± 9

[a]MAS, HT-2, NIV, and TOL were analyzed as their corresponding parent alcohols after alkaline hydrolysis.
[b]Mean +/- standard error; n = 6.

Figure II.38 Structures of selected trichothecenes and their corresponding parent alcohols.



Compound	Parent Alcohol[a]	R1	R2	R3	R4	R5
acetyl T-2	TOL	OAc[b]	OAc	OAc	H	Iso[c]
T-2 toxin	TOL	OH	OAc	OAc	H	Iso
HT-2	TOL	OH	OH	OAc	H	Iso
T-2 triol	TOL	OH	OH	OH	H	Iso
T-2 tetraol	TOL	OH	OH	OH	H	OH
neosolaniol	TOL	OH	OAc	OAc	H	OH
4-deacetylneosolaniol	TOL	OH	OH	OAc	H	OH
8-acetylneosolaniol	TOL	OH	OAc	OAc	H	OAc
triacetoxyscirpenol	STR	OAc	OAc	OAc	H	H
diacetoxyscirpenol	STR	OH	OAc	OAc	H	H
monoacetoxyscirpenol	STR	OH	OH	OAc	H	H
scirpentriol	STR	OH	OH	OH	H	H
deoxynivalenol	DON	OH	H	OH	OH	O
3-acetyl deoxynivalenol	DON	OAc	H	OH	OH	O
nivalenol	NIV	OH	OH	OH	OH	O
fusarenon-X	NIV	OAc	OH	OH	OH	O

[a]Parent Alcohols: TOL = T-2 tetraol; STR = scirpentriol; DON = deoxynivalenol; NIV = nivalenol.
[b]OAc = $OOCCH_3$.
[c]Iso = $OOCCH_2CH(CH_3)_2$.

Figure II.39 Chromatograms of extracts from soybean, corn, and mixed feeds naturally contaminated with trichothecenes. Amount of trichothecene parent alcohols detected: CORN--STR 105 ng/g, DON 2,630 ng/g, TOL 390 ng/g, SOYBEANS--DON 25 ng/g, TOL 185 ng/g, MIXED FEED--DON 210 ng/g, TOL 85 ng/g.

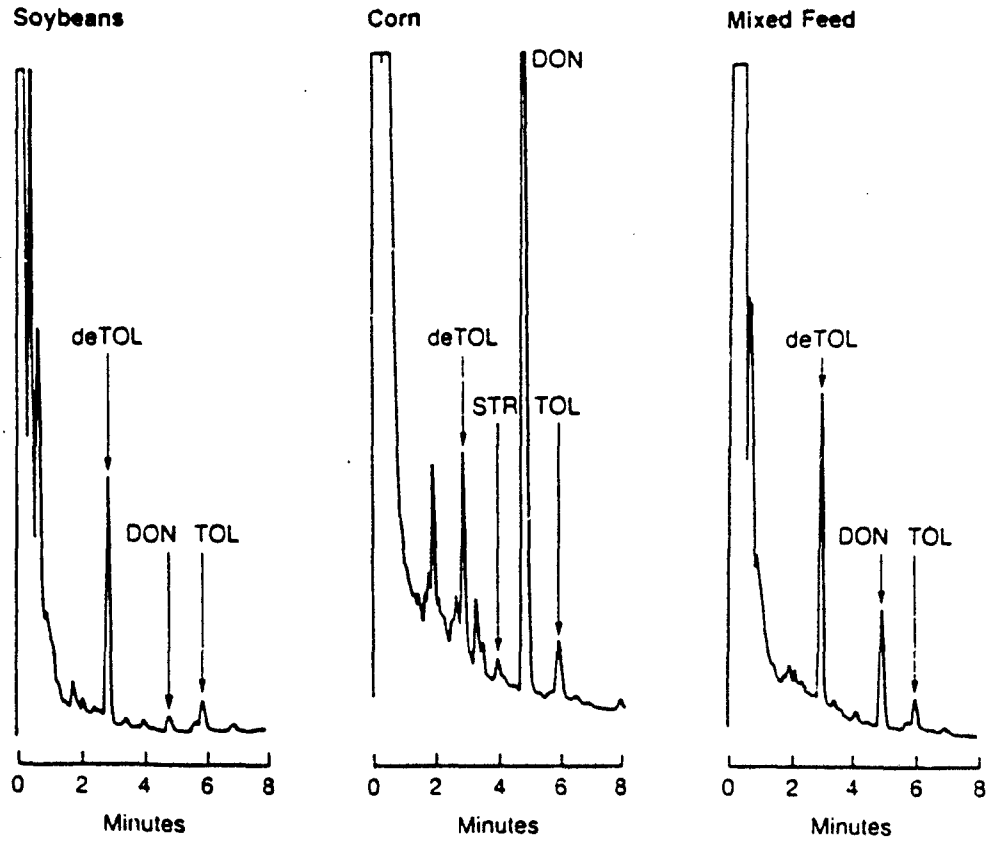
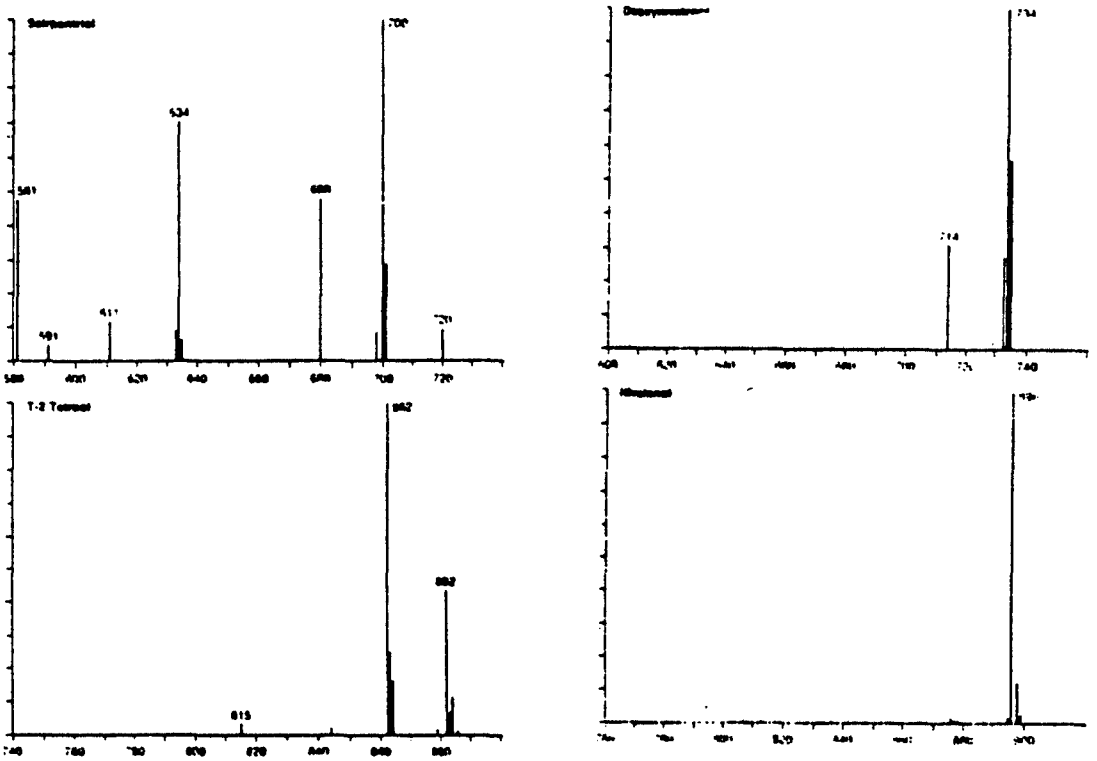


Figure II.40 Negative chemical ionization mass spectra of pentafluoropropioyl derivatives of the parent alcohols; scirpentriol, deoxynivalenol, T-2 tetraol and nivalenol.



2. Cross-reactivity of antibodies against T-2 with deepoxy T-2 toxin

by

Ru-Dong Wei, S. P. Swanson, and F. S. Chu

In cooperation with Dr. Ru-Dong Wei and Dr. F. S. Chu at the University of Wisconsin, deepoxy T-2 and deepoxy HT-2 were examined for cross-reactivity against 2 types of polyclonal anti-T-2 antibodies.

Abstract

Two types of antibodies raised against T-2 toxin, namely anti-T-2-HB-BSA and anti-3-Ac-NEOS-HS-BSA, showed good cross-reactivity with deepoxy T-2 toxin. Our results indicate that the epoxide is not an important epitope for the production of antibody against T-2 toxin.

Introduction

T-2 toxin, 4-β-15-diacetoxy-8-α-(3-methylbutyryloxy)-3-α-hydroxy-12,13-epoxytrichothec-9-ene, is one of the most toxic trichothecene mycotoxins produced by a number of species of Fusarium (Bamburg and Strong, 1971). In vivo and in vitro studies on the metabolism of T-2 toxin have led to the following pathways which may play a significant role in the toxicity of the toxin: a) hydrolysis of ester linkages (Ellison and Kotsonis, 1974; Knupp et al., 1986; Ohta et al., 1977; Visconti and Mirocha, 1985; Yoshizawa et al., 1981), b) hydroxylation of the C-3' position (Knupp et al., 1986; Wei and Chu, 1985; Yoshizawa et al., 1982; Yoshizawa et al., 1984), c) conjugation to form glucuronide (Corley et al., 1985), and d) deepoxidation of the C-12 and C-13 epoxide which is probably by deepoxidation of the parent toxins in the intestinal and/or rumen tracts (Swanson et al., 1987; Yoshizawa et al., 1986; Yoshizawa et al., 1985). In view of difficulties for the analysis of T-2 toxin and its metabolites, investigations in our laboratory as well as others have produced several antibodies against T-2 toxin and its metabolites (Chu et al., 1979; Fan et al., 1987; Hunter et al., 1985; Wei et al., 1986; Wei and Chu, 1987; Zhang et al., 1986). Several different types of immunoassays for trichothecene mycotoxins have been also developed (Chu, 1985). As the first step in development of an immunoassay for the newly discovered T-2 toxin metabolite deepoxy T-2 toxin, the search for specific antibodies was inevitable. In the present study, we have tested the cross-reactivity of two different types of antibodies against T-2 toxin that were raised in rabbits. One was obtained from rabbits after immunization with T-2-hemisuccinate-bovine serum albumin (T-2-HS-BSA, antibody A), and the other with 3-acetyl-neosolaniol-HS-BSA (3-Ac-NEOS-HS-BSA, antibody B). We found that both antibodies interact with the deepoxy T-2 toxin very effectively. Details of this study are reported below.

Experimental

T-2 toxin was prepared as described previously (Wei et al., 1971). Tritium-labeled T-2 toxin with a specific activity of 19 Ci/mM was prepared according to the procedure of Wallace et al. (1977). Deepoxy T-2 toxin was prepared using a modification of the method described by Colvin and Cameron (1986). Briefly, T-2 toxin was refluxed for 6 hours with 4 equivalents of tungsten hexachloride and 12 equivalents of n-butyl lithium. After concentration, the residue was redissolved in ethyl acetate and washed with 2 M sodium hydroxide solution. The organic layer

was washed with water and dried over anhydrous sodium sulfate. Purification of the resulting deepoxy T-2 toxin was accomplished by reversed phase HPLC in 55% methanol at a flow rate of 2 ml/minute (Alltech C_{18}, 10 μ column). Deepoxy T-2 toxin eluted in 70 to 74 ml. The deepoxy T-2 toxin fractions were combined, concentrated, and recrystallized in ethyl acetate/hexane. Deepoxy T-2 tetraol was synthesized by alkaline hydrolysis of deepoxy T-2 toxin and purified by reverse phase HPLC in 20% methanol. Purity was greater than 99.5% for both compounds as determined by capillary gas chromatography (Knupp et al., 1986) of the corresponding trimethyl silyl ether derivatives.

Anti-T-2-HS-BSA (Chu et al., 1979) and anti-3-Ac-NEOS-HS-BSA antibodies (Wei and Chu, 1987) were produced as described previously (Chu et al., 1979; Wei and Chu, 1987). Protocols for radioimmunoassay (RIA) were essentially the same as those described for T-2 toxin (Chu et al., 1979). In general, ^{3}H-T-2 toxin (ca. 12,000 dpm) was incubated with antiserum together with various concentrations of unlabeled T-2 toxin or its metabolites in 0.01 M phosphate-saline (0.16 M NaCl; PBS) at room temperature for 30 minutes and then in a cold room (6°C) for 1 hour or longer. Separation of bound and free ligand was achieved by an ammonium sulfate precipitation methods (Chu et al., 1979). The radioactivity of the free ligand was determined in a Beckman model LS-5801 liquid scintillation spectrometer using 5 ml of Aquasol (New England Nuclear Corp., Boston, MA).

Results and Discussion

In the present study, anti-T-2 toxin antibodies obtained from rabbits after immunization with 2 different immunogens (Figure II.41) derived from T-2 toxin were selected to test their cross-reactivity with deepoxy T-2 toxin. Although both antibodies have high affinity for T-2 toxin, antibody A is highly specific for T-2 toxin (Chu et al., 1979), whereas antibody B has a wide specificity and cross-reacts with a number of group A trichothecene mycotoxins (Wei and Chu, 1987). Results for the cross-reactivity of these 2 antibodies with deepoxy T-2 toxin, as analyzed by a competitive RIA, are shown in Figure II.42. Both antisera show good cross-reactivities with deepoxy T-2 toxin. The concentration causing 50% inhibition of binding of ^{3}H-T-2 toxin to antibody A by unlabeled T-2 and deepoxy T-2 toxin were 0.18 ng and 0.23 ng/assay, respectively. For antibody B, the concentration causing 50% inhibition of binding of ^{3}H-T-2 toxin by T-2 toxin and deepoxy T-2 toxin were 0.35 ng and 0.7 ng/assay, respectively. Deepoxy T-2 tetraol did not inhibit the binding of both antibodies at 2,000 ng/assay. Thus, the binding of deepoxy T-2 toxin with both antibodies is similar to binding with T-2, especially with antibody A. Because both antibodies showed good cross-reactivity with the deepoxy T-2 toxin, our results suggest that the epoxide functional group in T-2 toxin did not contribute to the antigen-antibody recognition. These results are consistent with earlier observations on the physicochemical data of the T-2 toxin structure (Sigg et al., 1965) which suggests that the epoxide group in the T-2 toxin is shielded sterically to the extent that the usual reagents do not attack under mild conditions. Present results also suggest that it is not necessary to produce specific antibody for the deepoxy toxin.

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Figure II.41 Structures of 2 immunogens used for the production of antibodies against T-2 toxin: A for antibody A (anti-T-2-HS-BSA), B for antibody B (anti-3-Ac-NEOS-HS-BSA).

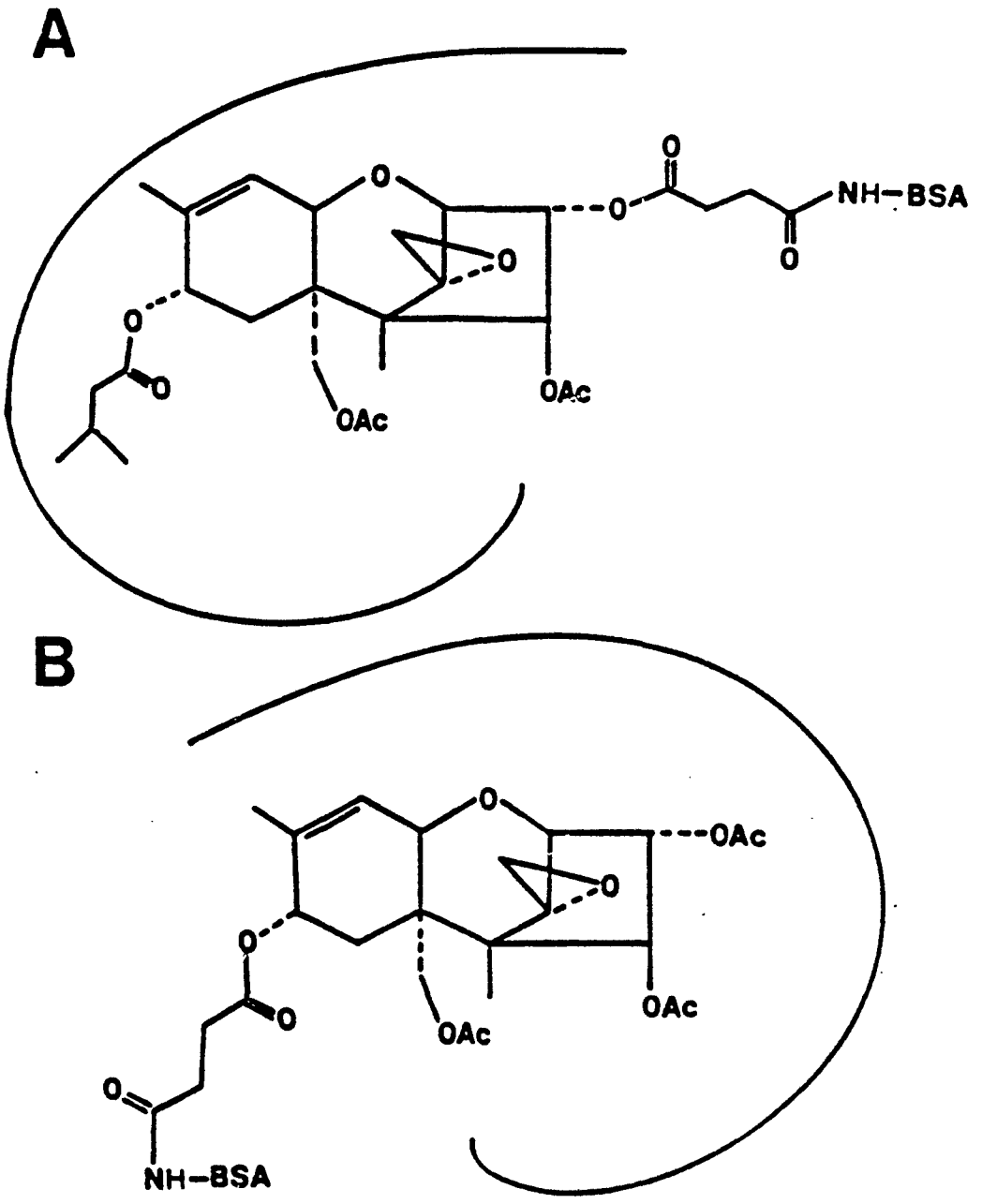
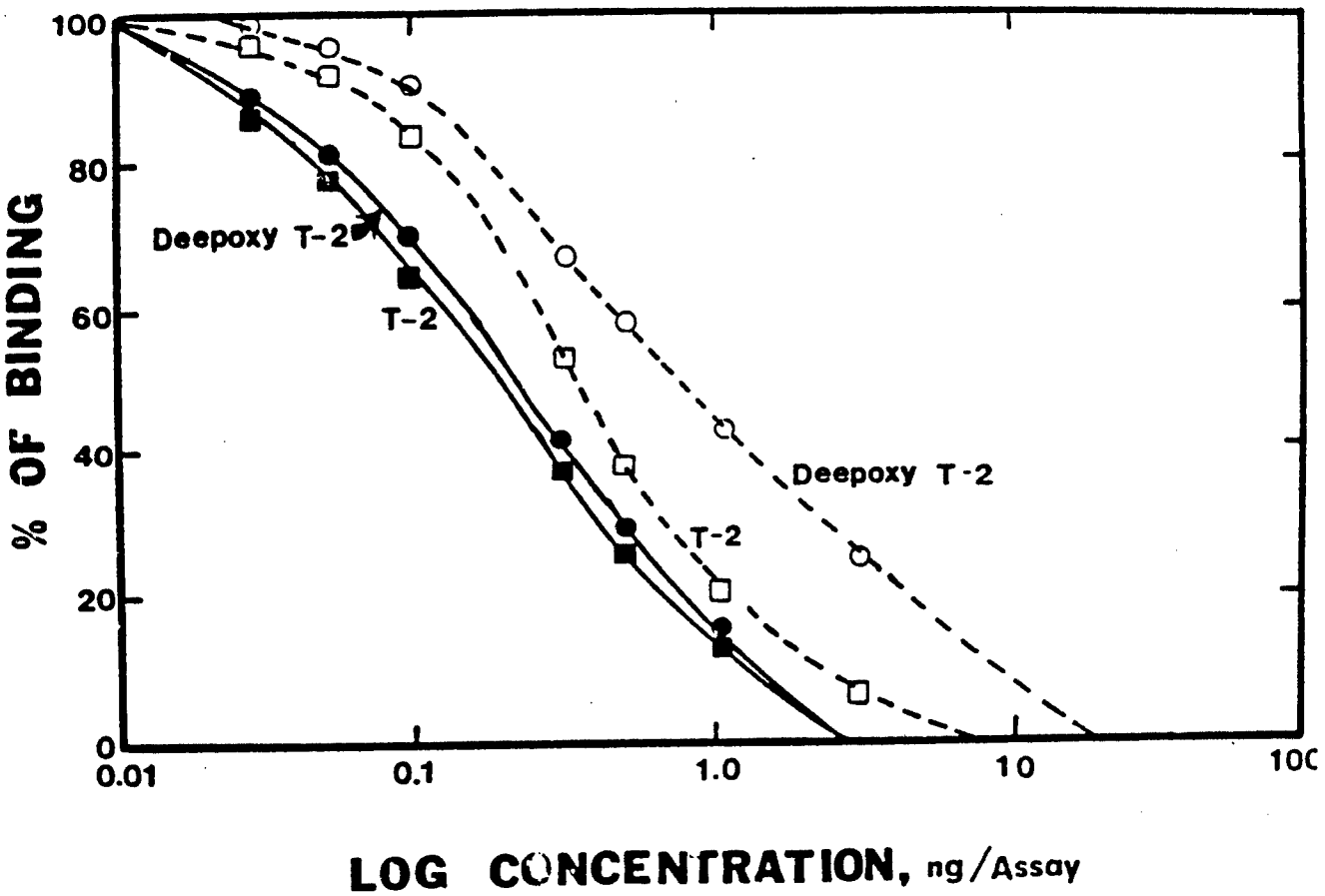


Figure II.42 Effect of T-2 and deepoxy T-2 toxins on the binding of ^{3}H-T-2 toxin with rabbit anti-T-2-HS-BSA (——) and anti-3-Ac-NEOS-HS-BSA (----).



3. Cross-reactivity of T-2 metabolites to polyclonal and monoclonal anti-T-2 antibodies

The binding specificities of 2 different types of anti-T-2 antibodies (monoclonal and polyclonal) to several T-2 metabolites has been previously reported (Swanson et al., 1987). Data is summarized in Table II.21.

Wei et al. (see previous section) examined 2 different anti-T-2 polyclonal antibodies for cross-reactivity with deepoxy T-2 and deepoxy TOL. With both antibody types, cross-reactivity of deepoxy T-2 was similar to T-2 toxin, whereas neither antibody cross reacted to any significant extent with deepoxy tetraol. The polyclonal antibody (developed by Biometric Systems Incorporated) shown in Table II.21 exhibited limited cross-reactivity to deepoxy HT-2. This antibody, however, was not examined for cross-reactivity with deepoxy T-2. In contrast to the polyclonal antibody, monoclonal antibody (15H6) displayed approximately twice the binding activity to HT-2 and deepoxy HT-2 as it did to T-2 toxin. Both studies indicate that the epoxide moiety in trichothecene mycotoxins is not an essential epitope for antibody recognition.

These data demonstrate that antibodies against trichothecenes can vary greatly, not only in their sensitivity but also their cross-reactivity to related metabolites. Therefore, all trichothecene antibodies should be evaluated for cross-reactivity to a variety of metabolites for accurate results to be obtained. However, in certain circumstances, strong cross-reactivity to trichothecene metabolites could be advantageous. Since deepoxidation and hydroxylation are major biotransformation pathways, antibodies which cross-react with these and other metabolites would be useful for screening samples for suspected exposure to trichothecenes. This cross-reactivity is especially advantageous for this purpose when one considers the plasma disappearance half lives of T-2 toxin and DAS are extremely short and little parent compound can be detected in blood or urine of animals exposed to these toxins.

Deepoxy T-2 is a non-toxic trichothecene. Once the epoxide group has been reduced to a carbon-carbon double bond, toxicity is eliminated. Since deepoxy T-2 cross reacts with anti-T-2 antibodies nearly equally to T-2 toxin itself, deepoxy T-2 could serve as a nontoxic quality control agent for immunoassay screening tests. In this manner enzyme viability could be documented as part of quality control check when analyzing samples, without requiring the use of the potent cytotoxic T-2 toxin.

Immunoassays offer many advantages for rapid screening of samples suspected of containing trichothecenes, including speed, decreased cost and sensitivity. However, immunoassays are only screening techniques, and all positive samples should be confirmed by other techniques such as GC-MS. Since deepoxy trichothecenes cross-react with most anti-T-2 antibodies examined to date, it is important to include deepoxy metabolites in the confirmational GC-MS analysis of positive immunoassay samples of blood or urine.

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Table II.21 Cross-reactivity comparison of epoxy and deepoxy metabolites to polyclonal and monoclonal T-2 antibodies.

Compound	% Cross-Reactivity[a] of Polyclonal T-2[b]	Monoclonal T-2[c]
T-2	100	100
HT-2	17.4	225
DEHT-2	14.6	189
TRIOL	0.5	0.2
DE TRIOL	1.8	9.2
TETRAOL	<< 1	<< 1
3'OH T-2	0.4	0.4
3'OH HT-2	<< 1	<< 1

[a]Relative binding cross-reactivity of trichothecene metabolites to the antibodies compared with T-2 toxin (ng of T-2 required to displace tritium-labeled T-2 by 50% ÷ ng of metabolite required to displace tritium-labeled T-2 by 50%) x 100.
[b]Rabbit polyclonal T-2 antibodies.
[c]Mice monoclonal T-2 antibody strain 15H6.

4. Analysis of plasma and urine for trichothecenes

The trichothecene mycotoxins T-2, DAS, and DON are extensively metabolized by a variety of animal species. Many metabolites of T-2 and DAS have been detected in the blood, urine, feces, bile, and tissues of exposed animals including swine (Bauer et al., 1985; Corley et al., 1985,1986), poultry (Chi, et al., 1978; Visconti et al., 1985a,b; Yoshizawa et al., 1980; Yoshizawa et al., 1982), rats (Ohta et al., 1978; Pace, 1986; Sakamoto et al., 1986), and cattle (Pawlosky et al., 1984; Yoshizawa et al., 1981). It has been recently reported that several deepoxide metabolites of T-2, DAS, and DON have been found in rats, pigs, and cattle (Chatterjee et al., 1986a,b; Corley et al., 1986; Côté et al., 1986a,b; Sakamoto et al., 1986; Swanson, 1987; Yoshizawa et al., 1983,1985).

Trichothecene metabolites exhibit a wide range of chemical behavior due to the varied number and types of side groups. This is especially true when one considered the large number of metabolites detected in animals experimentally administered trichothecenes. Current methods for chemical analysis of T-2, DAS, DON, or NIV, along with their metabolites, in biological samples can be lengthy or difficult especially, if all metabolites are monitored. Standards of many of the metabolites are not commercially available and may be costly or difficult to synthesize or maintain.

One approach we have developed in an effort to minimize the problems described above is to hydrolyze all trichothecenes to their parent alcohols prior to analysis. With this approach, relatively few standards are required and the majority are commercially available. A brief overview of the method is as follows. Samples of plasma or urine are extracted with a C-18 cartridge. The concentrated eluate is further purified on a florisil minicolumn. The trichothecene residues present are then hydrolyzed to their parent alcohols under alkaline conditions, separated on a silica cartridge derivatized, and quantitated by capillary GC-ECD. Confirmation is accomplished by GC-MS using negative chemical ionization. See Rood et al. (1988) for details of the method. A similar approach can be used for analysis of feed samples.

The advantage of this approach is that the method is relatively rapid for confirming exposure of animals to trichothecenes. Only 4 standards (T-2 tetraol [TOL], scirpentriol [STR], deoxynivalenol [DON], and nivalenol [NIV]) are necessary to screen for T-2, DAS, DON, or NIV exposure in a single analysis, and the standards are readily available through several chemical supply houses. For confirmation, gas chromatography/mass spectroscopy can be performed with little or no additional workup of the samples extracted for gas chromatographic analysis. Disposable glassware is used throughout the method to eliminate the possibility of contamination or carry-over from previous samples.

In this method, T-2 and its metabolites are converted to TOL, and DAS and its metabolites are converted to STR with 0.1 N NaOH; DON and NIV do not have ester side groups and are unchanged under these conditions. TOL, STR, DON, and NIV do not contain any ester groups and are referred to as the "parent alcohol" for each of their respective trichothecene group. Table II.22 shows the various metabolites and their corresponding parent alcohol formed after alkaline hydrolysis. The hydrolysis conditions utilized were selected to maximize the conversion of T-2 to TOL and DAS

to STR and to minimize the degradation of DON and NIV. The detection of TOL, STR, DON, NIV, or their deepoxy analogs in a sample would indicate trichothecene exposure.

Another advantage to this approach in screening samples is that conversion of metabolites to their parent alcohols by alkaline hydrolysis leads to a cumulative effect. This results in a greater sensitivity in detecting trichothecene exposure since several different metabolites are converted to a single, corresponding parent alcohol (i.e., T-2 plus metabolites → TOL; DAS plus metabolites → STR). Also, upon derivatization to their corresponding fluoroacyl derivatives, the parent alcohols exhibit a significantly greater response factor by both GC-ECD and NCI GC-MS compared to the esterified metabolites. The detection limit is better than 25 ng/ml for TOL, STR, DON, and NIV.

Recently, many deepoxy metabolites of T-2, DAS, and DON have been detected as metabolites in the urine and plasma of rats, pigs, or cattle administered trichothecenes (Chatterjee et al., 1986; Corley et al., 1986; Côté et al., 1986a,b; Pfeiffer et al., 1988; Swanson et al., 1988; Yoshizawa et al., 1980b; Yoshizawa et al., 1983; Yoshizawa et al., 1985; Yoshizawa et al., 1986). Using the hydrolysis method approach, deepoxy metabolites of T-2 are converted to deepoxy T-2 tetraol (DE TOL), and the deepoxy metabolites of DAS are converted to deepoxy scirpentriol (DE STR); DOM-1, the deepoxy metabolite of DON, behaves similar to DON. Detection of a deepoxy analog of a parent alcohol (DE TOL, DE STR, or DOM-1) would also indicate trichothecene exposure.

One final advantage of this method which analyzes for the parent alcohols after alkaline hydrolysis rather than the parent compounds such as T-2 toxin, DAS, and/or individual metabolites. T-2 and DAS plasma levels drop rapidly following the administration of an i.v. dose. T-2 toxin plasma disappearance half-lives of 10 to 20 minutes in the blood of pigs (Beasley et al., 1986) and 5 to 10 minutes in the blood of dogs (Sintov et al., 1986) have been reported. Generally, metabolites of T-2 or DAS are present in blood or urine long after the parent compounds have dropped to nondetectable concentrations. This screening method can detect the presence of these metabolites at low levels in an easy and relatively rapid manner including confirmation by GC-MS. Trichothecene exposure can be determined long after the parent compound(s) can no longer be detected.

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Table I.22 Identity of parent alcohols after hydrolysis with NaOH.

T-2 T-2 triol HT-2 T-2 tetraol Neosolaniol 4-deacetylneosolaniol 3'OH T-2 3'OH HT-2 3'OH TRIOL 4-acetyl tetraol 8-acetyl tetraol	OH- →	T-2 tetraol
Diacetoxyscirpenol Monoacetoxyscirpenol Scirpentriol	OH- →	scirpentriol
Deoxynivalenol 3-acetyl deoxynivalenol 15-acetyl deoxynivalenol	OH- →	deoxynivalenol
Nivalenol Fusarenon-X	OH- →	nivalenol
Deepoxy HT-2 Deepoxy triol Deepoxy tetraol Deepoxy 3'OH HT-2	OH- →	deepoxy tetraol
Deepoxy monoacetoxyscirpenol Deepoxy scirpentriol	OH- →	deepoxy scirpentriol
Deepoxy deoxynivalenol	OH- →	deepoxy deoxynivalenol

5. Confirmation of trichothecenes exposure

In situations where toxin exposure is uncertain, confirmation of samples found to be positive by GC-ECD or immunoassay techniques are essential. For all practical purposes the only suitable means for confirmation utilizes mass spectrometry. However, several different types of mass spectrometry are available for use including: GC-MS electon impact, GC-MS positive chemical ionization, GC-MS negative chemical ionization, thermospray HPLC-MS positive ionization, and thermospray HPLC-MS negative ionization. In addition, with GC-MS there is the choice of injection modes for derivatized or underivatized sample extracts, in addition to several different derivatives to choose from for GC-MS with electron impact, positive CI, and negative CI ionization techniques. In addition, multiple MS techniques such as GC-MS/MS are now available in a few laboratories. As the later is a specialized technique requiring expensive instrumentation not available to most laboratories, MS/MS will not be discussed further.

Electron Impact GC-MS

Although electron impact GC-MS is the most commonly used form of ionization for mass spectrometry systems, when applied specifically to the trichothecenes there are several inherent disadvantages. The most important of which is the lack of sensitivity. Trichothecenes in general fragment to a significant extent under electron impact ionization. In most cases no moleculoar ion is observed or the molecular ion is very small and undistictive. This is particularly true with derivatized toxins, both the fluorinated ester derivatives and the trimethylsilyl ether (TMS) derivatives of trichothecenes. As a result of the extensive fragmentation under electron impact ionization, sensitivity is reduced and fragment ions are of a lower molecular weight. Therefore, GC-MS with electron impact ionization of the trichothecenes has been replaced for the most part by other forms of ionization techniques.

Negative Chemical Ionization

Negative chemical ionization (NCI) of trichothecenes provides the greatest sensitivity in detection. Under ideal conditions with clean samples, as littles as 25 pg of fluorinated ester derivatives of the trichothecene parent alcohols can be detected by GC-MS NCI. In most instances the molecular ion or M-H ions are the predominant or only fragments observed. Toxins such as T-2 or DAS which have only a single hydroxyl group and therefore form a derivative with a single fluorinated acyl group display a reduced sensitivity compared compounds such as T-2 tetraol, DON, and Nivalenol which form multi-fluoroacyl derivatives.

One major advantage of negative chemical ionization is that it is the only GC-MS technique which approaches the sensitivity of GC with electron capture detection. In most instances, NCI mass spectra can be obtained from samples found positive by GC-ECD with no additional workup of the sample other than a simple concentration step. Many groups, including our laboratory, have successfully used GC-MS NCI for confiration of samples found positive by GC-ECD screening methods. See Annual Report submitted December 30, 1986, and section IA above for mass spectra of derivatized trichothecenes we have obtained and details on experimental conditions.

Although GC-MS NCI is the most sensitive mass spectral confirmation technique for trichothecenes, it does suffer from one potential disadvantage. The high sensitivity of NCI results at least in part due to the lack of fragmentation. The molecular ion or M-H is typically the only or the predominent ion detected and only limited structural information on the molecule in question is obtained. In addition, GC-MS NCI is frequently used when the sample concentrations are very low, approaching the lower sensitivity of the instrument. As a result, confirmation of trichothecenes in samples at low concentrations and present in complex matrices must theoretically be viewed with a certain degree of caution when using GC-MS NCI in order to eliminate potential false positives.

Thermospray HPLC-MS

Thermospray HPLC MS offers several advantages not available by other MS techniques such as GC-MS. The major advantage of HPLC MS is that derivatization of the toxins is avoided and less sample preparation is required compared to GC-MS analysis. It is also possible to analyze conjugates directly, without enzymatic hydrolysis or derivatization. Hence HPLC MS can provide a single-step metod for the confirmation of trichothecenes and other toxins, in addition to their conjugated metabolites, in biological fluids. We have successfully utilized HPLC-MS for the analysis of trichothecene mycotoxins in feces, plasma, and urine samples. See Voyksner et al., 1987, and DAMD 17-85-C-5224 annual report dated December 30, 1986, for further details.

As with GC-MS, HPLC-MS can be operated in both positive and negative detection modes. Thermospray mass spectra of trichothecene mycotoxins showed $[M + H]+$ ions plus numerous structurally significant fragment ions in the positive detection mode. Generally the toxins displayed and $M + H$ and/or $M + NH_4$ with loss of H_2O, CH_3COO, and CH_2. The negative ion spectra of the toxins displayed limited fragments, giving only molecular weight information. The major anions observed were $M + CH_3COO$ and $M - H$ or $M -$.

Spectra obtained under chemical ionization (CI) with the filament on were nearly identical to those obtained under thermospray ion evaporation conditions for both negative and positive spectra. However, CI proved to be several orders of magnitude greater in sensitivity. Negative ion detection CI thermospray was the most sensitive method. Detection limits of 0.05 to 0.50 ng per injection were obtained for the trichothencenes and their metabolites using multiple ion detection.

In conclusion, thermospray HPLC-MS is a viable alternative to GC-MS when the instrumentation is available. This technique also offers several distinct advantages over GC-MS in that derivatization is not required prior to analysis and polar nonvolatile compounds such as glucuronide conjugates of toxins may be detected in biological matrices directly.

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D. Production and Characterization of Trichothecene Metabolites

As previously stated in this document, the metabolism of trichothecenes occurs via four basic biotransformation pathways: 1) hydrolysis of esters, 2) oxidation (hydroxylation), 3) epoxide reduction (deepoxidation) and 4) glucuronide conjugation. In the course of this contract we have produced and purified many metabolites of T-2 toxin and DAS, which result from the first three biotransformation pathways and which are unavailable through commercial channels.

During the course of this contract, ten different trichothecene metabolites were produced, purified and sent to USAMRIID. These compounds can be segregated into 4 different groups: 1) deepoxy metabolites of T-2, 2) deepoxy metabolites of diacetoxyscirpenol, 3) oxidation products of T-2 and 4) deacylation product of T-2.

In all cases the compounds involved were known to be animal metabolites of T-2 toxin or DAS, with the exception of deepoxy T-2. A summary of the compounds and the amounts forwarded to USAMRIID are given below.

Summary of compounds produced under this contract and supplied to USAMRIID.

Compound	Amoung (mg)	Reference
DE T-2	50	Swanson et al., 1988
DE HT-2	100	Swanson et al., 1987a
DE TOL	100	Swanson et al., 1987a
DE MAS	100	Swanson et al., 1987a
DE SCP	100	Swanson et al., 1987a
3'OH T-2	100	Knupp et al., 1987a
3'OH HT-2	100	Knupp et al., 1987a
3'OH TRIOL	0.5	Knupp et al., 1987a
4'OH T-2	1.25	Knupp et al., 1987b
4-DN	10	Yoshizawa et al., 1980; Knupp 1987a

Capillary gas chromatograms of the corresponding trimethylsilyl (TMS) ether derivatives, in addition to positive chemical ionization mass spectra, were provided with the samples. With all samples sent to USAMRIID, purity was determined to be greater than or equal to 95% as determined by capillary GC and thin-layer chromatography.

Different production schemes were used for the production of these trichothecene metabolites depending upon the different site(s) on the parent compound which were altered. In several instances multiple steps were required. A brief description of the methods used to prepare these compounds are given below.

1. Products resulting from microsomal oxidation

Production of 3'-hydroxy T-2 toxin and 3'-hydroxy HT-2. The production of purified 3'-hydroxy T-2 toxin and 3'-hydroxy HT-2 toxin were readily accomplished using liver S-9 (9,000 x g liver homogenate supernatant fraction) preparations and T-2 toxin as the starting material.

In addition to 3'OH T-2 and 3'OH'HT-2, 3'OH TRIOL and 4'OH T-2 were also formed in minor quantities. For production of 3'OH T-2 and 4'OH T-2, oxidized products retaining the C-4 ester group, the addition of paraoxon as an esterase inhibitor was essential. The addition of paraoxon reduced hydrolysis of the C-4 and C-15 acetyl groups, thereby increasing the overall yields. In addition, fewer metabolites were produced hence purification was less complex. Details of experimental procedures for the production and purification of the oxidation products can be obtained from Knupp et al., 1987a,b and DAMD 17-85-C-5224 Annual Report submitted December 30, 1986 (p. 328).

2. Products resulting from deepoxidation reactions

Deepoxidation products of T-2 toxin and diacetoxy scirpenol (except for DE T-2) were most efficiently produced using a biosynthetic route with anaerobic gastrointestinal microorganisms. Initial work was accomplished through the use of rumen microflora as inoculum. Although the desired products were produced (DE HT-2, DETKIOL, DE MAS, DE SCP), yields were lower than expected and the media was very complex resulting in the need for extensive purification. Results from studies described earlier in this report on the comparative metabolism of T-2 and DAS by fecal microflora from several species, indicate rat cecal microflora are the most efficient in reducing the epoxide group in trichothecenes to a carbon-carbon double bond.

Deepoxy T-2 could only be produced via a synthetic route using tungsten hexachloride and n-butyl lithium. Although we were able to achieve yields up to 50% on a small scale, when the process was scaled up (> 0.1 mmol) yields decreased. In addition, the major by-product was unreacted parent T-2 toxin. Separation of DE T-2 from T-2 toxin required a lengthy reverse phase HPLC purification step in order to obtain deepoxy T-2 which was $\geq$ 99% pure and more importantly, free of any contaminating T-2 toxin.

Details on experimental procedures for the production and purification of deepoxy trichothecenes can be obtained in Swanson et al., 1987, 1988. Capillary GC retention times of the TMS and TFA derivatives of these trichothecenes are given in Tables II.23 to II.25. A summary of the schemes used for production of the trichothecene metabolites sent to USAMRIID are given below.

3. Production schemes for major deepoxy trichothecene metabolites

a. Deepoxy compounds of T-2 toxin

(1) Deepoxy T-2 (DE HT-2)

$$\text{T-2} \xrightarrow[WCl_6]{n\text{BuLi}} \text{DET-2}$$

(2) Deepoxy HT-2 (DE HT-2) and deepoxytriol (DE TRIOL)

$$\text{T-2} \xrightarrow[\text{anaerobically}]{\text{GI microflora}} \text{DE HT-2(major)} + \text{DE TRIOL(minor)}$$

or

$$\text{DE T-2} \xrightarrow{NH_4OH} \text{DE HT-2} + \text{DE TRIOL} + \text{DE TOL}$$

(3) Deepoxy Tetraol (DE TOL)

$$\text{DE T-2(from synthesis)} \xrightarrow{\text{NaOH}} \text{DE TOL}$$

or

$$\text{DE HT-2(from GI incubation)} \xrightarrow{\text{NaOH}} \text{DE TOL}$$

(4) Deepoxy 3'OH HT-2 (DE 3'HT-2)

$$\text{step 1} \quad \text{T-2} \xrightarrow[\text{NADPH}]{\text{Liver S-9}} \text{3'OH HT-2}$$

$$\text{step 2} \quad \text{3'OH HT-2} \xrightarrow[\text{anaerobically}]{\text{GI Microflora}} \text{DE 3'OH HT-2}$$

b. Deepoxy deoxynivalenol (DOM-1)

$$\text{DON} \xrightarrow[\text{anaerobically}]{\text{GI Microflora}} \text{DE DON (DOM-1)}$$

c. Deepoxy metabolites of diacetoxyscirpenol

(1) Deepoxy DAS (DE DAS)

$$\text{DAS} \xrightarrow[WCl_6]{n\text{BuLi}} \text{DE DAS}$$

(2) Deepoxy monoacetoxyscirpenol (DE MAS)

$$\text{DAS} \xrightarrow[\text{anaerobically}]{\text{GI Microflora}} \text{DE MAS} + \text{DE SCP}$$

(3) Deepoxyscirpentriol (DE SCP)

$$\text{DE MAS} \xrightarrow{\text{NaOH}} \text{DE SCP}$$

4. Production of 3'-hydroxylated metabolites of T-2 toxin

   a. 3'-OH T-2

   T-2 ---(Liver S-9, NADPH, paraoxon)---> 3'-OH T-2

   b. 3'-OH HT-2 and 3'-OH TRIOL

   T-2 ---(Liver S-9, NADPH)---> 3'-OH T-2 + 3'OH HT-2 + 3'OH TRIOL

   or

   3'-OH T-2 ---(NH_4OH)---> 3'OH HT-2 + 3'OH TRIOL + TOL

   c. 4'-OH T-2

   T-2 ---(Liver S-9, NADPH, paraoxon)---> 4'-OH T-2(minor) + 3'-OH T-2

5. Production of miscellaneous trichothecenes

   a. Neosolaniol (NEO)

   F. sporotrichioides NRRL 3299 (room temperature incubation)

   b. 4-deacetylneosolaniol (4 DN)

   scheme a F. sporotrichioides NRRL 3299

   scheme b NEO ---(NH_4OH)---> 4-DN + TOL + 15-DN

   scheme c T-2 ---(Liver S-9)---> HT-2 + 4-DN + TOL + TRIOL

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Table II.23 Retention times of trichothecene mycotoxins as their corresponding trifluoroacetyl ester derivatives.

Compound	Concentration (µg/µl)	RT (min)	RRT
C_{28} (ISTD)	0.500	9.075	1.000
DE TETRAOL	0.100	2.581	0.287
TETRAOL	0.100	3.735	0.415
4-DN	0.050	4.362	0.483
DE TRIOL	0.180	4.572	0.509
DE HT-2	0.100	5.772	0.643
TRIOL	0.100	6.165	0.685
HT-2	0.100	7.573	0.841
T-2	0.300	10.683	1.187
3'HT-2*	0.100	7.821	0.871
		8.562	0.953
3'T-2*	0.100	10.945	1.218
		11.940	1.329
NEO	0.100	(not reproducibly derivatized)	
NIV	0.100	2.802	0.311
DOM-1	0.100	3.080	0.340
DON	0.100	3.345	0.369
FUS-X	0.100	4.120	0.458
3Ac-DN	0.050	5.300	0.587
DE STRIOL	0.100	2.212	0.243
DE MAS	0.250	2.972	0.326
STRIOL	0.104	3.035	0.333
MAS	0.200	4.235	0.465
DAS	0.417	6.478	0.711

*Split peaks are found for 3'HT-2 and 3'T-2 using TFAAn derivative. The first Retention Time listed is the larger peat of the two.

PARAMETERS AS BELOW:

Hewlett Packard 5790 GC with flame ionization detection
Column: 30 m x 0.25 mm id, 0.25 µ DB 1701 capillary column
Initial temperature: 225°C
Final temperature: 275°C
Final hold: 12.0 minutes
Rate: 5°C/minute
Detector temperature: 300°C
Injector temperature: 275°C
Carrier gas: hydrogen with linear velocity of 45 cm/second

Table II.24 Retantion times of trichothecenes and their various metabolites by capillary GLC/FID.

Compound	Concentration (µg/µl)	RT (min)	RRT
$C_{30}H_{62}$ (ISTD)	.20	8.639	1.000
deepoxy Tetraol		2.518	.292
Tetraol	.50	4.011	.464
deepoxy Triol		5.456	.633
4-DN	.50	6.151	.712
Triol	.50	7.555	.874
Neosolaniol	.50	7.569	.876
deepox HT-2	.25	7.581	.879
3'-OH Triol	.25	9.691	1.124
HT-2	.50	9.857	1.141
T-2	1.00	11.483	1.329
3'-OH HT-2	.50	12.063	1.396
4'-OH HT-2		12.717	1.477
3'-OH T-2	.50	13.960	1.616
Acetyl T-2	.33	14.28	1.659
4'-OH T-2	1.00	14.766	1.709
DOM-1	.10	2.399	.278
DOn	.25	3.759	.436
Nivalenol	.25	4.880	.566
deepoxy STR	.25	2.177	.252
deepoxy MAS	.25	3.302	.383
Scirpenetriol	.25	3.539	.411
MAS	.10	5.113	.593
DAS	.50	6.804	.788
TAS		9.453	1.093
Zearalenone	.50	12.11	1.421
Isoneo		6.52	
Fusarenon X	.352	4.77	.561
Zearalenol	.732	10-50	1-235

Column: DB-17, 15 m x 0.32 mm id x 0.25 µ film
Hydrogen carrier gas: linear velocity 48 cm/second
Program: 200°C to 260°C at 5°C/minute, hold 260

Table II.25 Retention times (RT) of the corresponding TMS ether derivatives of trichothecene mycotoxins.

Compound	Concentration (µg/µl)	RT (min)	RRT[a]
C_{30} (ISTD)	0.5	7.046	1.000
DE TETRAOL	0.125	2.628	0.373
TETRAOL	0.250	3.708	0.526
DE TRIOL	0.225	4.464	0.634
4-DN	0.250	4.835	0.689
NEO	0.453	5.532	0.762
DE HT-2	0.125	5.685	0.807
TRIOL	0.250	5.958	0.846
DE 3'TRIOL	0.225	6.109	0.867
DE T-2	0.500	6.500	0.927
HT-2	0.250	7.545	1.071
DE 3'OH HT-2	0.500	7.987	1.127
T-2	0.750	8.284	1.176
3'TRIOL	0.500	8.446	1.188
3'OH HT-2	0.220	10.783	1.530
3'OH T-2	0.145	11.999	1.712
4'OH T-2	0.200	13.10	1.869
DOM-1	0.240	2.429	0.346
DON	2.000	3.022	0.431
FUS-X	0.352	3.655	0.521
NIV	0.455	3.736	0.531
3Ac-DN	0.250	4.165	0.593
DE STRIOL	0.300	2.305	0.329
DE MAS	0.750	2.842	0.405
STRIOL	0.310	3.102	0.443
MAS	0.600	3.857	0.550
DAS	1.250	4.452	0.635

[a]Relative retention time.

PARAMETERS AS BELOW:

Column: 30 m x 0.25 mm id, 0.25 µ DB 1701 capillary column
Flow rate: 48 cm/second, hydrogen
Temperature program: initial 250°C
final 275°C
final hold 6 minutes
rate 5°C/minute
Detector temperature: 300°C
Injector temperature: 275°C

Publications Supported in Part by Army Contract Funds

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RJL:11b/14
10/03/88