

REPORT

FINAL REPORT

Task 97-53: Protection

of Guinea Pigs by Passive

Immunization with Human

Botulinum Immune

Globulin Obtained Post

Primary Series and Post

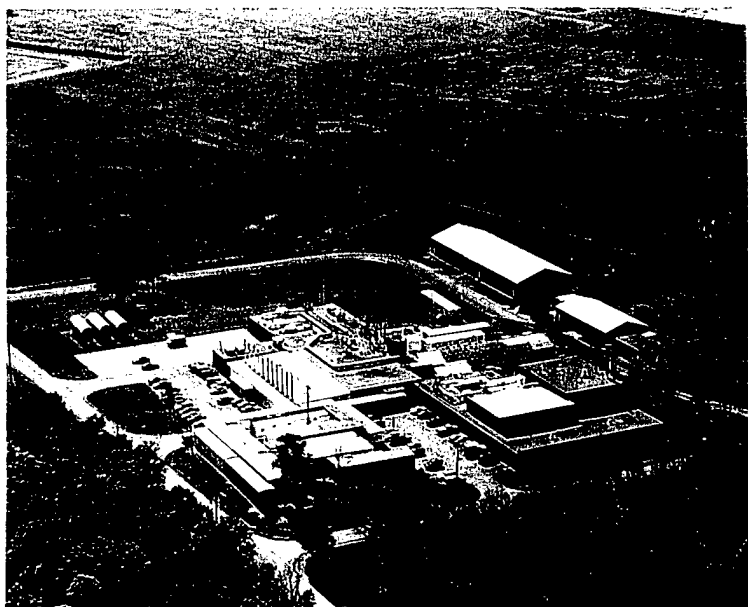
Six-Month Booster

Immunization

To

Joint Program Office for Biological
Defense

January 2001



AD _____

Award Number: DAMD17-89-C-9050

TITLE: A Medical Research and Evaluation Facility (MREF) and
Studies Supporting the Medical Chemical Defense Program

SUBTITLE: Protection of Guinea Pigs by Passive Immunization with
Human Botulinum Immune Globulin Obtained Post Primary
Series and Post Six-Month Booster Immunizations

PRINCIPAL INVESTIGATOR: Carl T. Olson, Ph.D., Robert E. Hunt,
Rebekah A. Starner, Michelle L. Clagett,
Nancy A. Niemuth, M. Claire Matthews

CONTRACTING ORGANIZATION: Battelle
Columbus, Ohio 43201-2693

REPORT DATE: October 2000

TYPE OF REPORT: Final, Task 97-53

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution unlimited

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.

20010312 147

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 2000	3. REPORT TYPE AND DATES COVERED Final, Task 97-53	
4. TITLE AND SUBTITLE A Medical Research and Evaluation Facility (MREF) and Studies Supporting the Medical Chemical Defense Program Subtitle: Protection of Guinea Pigs by Passive Immunization with Human Botulinum Immune Globulin Obtained Post Primary Series and Post Six-Month Booster Immunizations			5. FUNDING NUMBERS DAMD17-89-C-9050	
6. AUTHOR(S) Carl T. Olson, Ph.D., Robert E. Hunt, Rebekah A. Starner, Michelle L. Clagett, Nancy A. Niemuth, M. Claire Matthews				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Battelle Columbus, Ohio 43201-2693 E-MAIL: olsonc@battelle.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; Distribution unlimited.				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) See report.				
14. SUBJECT TERMS Nerve Agents, Antidotes, Chemical Agents, CSM, Sheep, Vesicants, Decontamination, Skin Protection, Desert Shield, BL3, Biodefense, Advanced Development, RADV, Chemical Defense, RADIV				15. NUMBER OF PAGES 396
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

TABLE OF CONTENTS

Page

TABLE OF CONTENTS	
1.0 INTRODUCTION.....	1
2.0 MATERIALS AND METHODS	3
2.1 Animals	3
2.2 Antibody Preparations.....	4
2.3 Toxin Preparations	5
2.4 Experimental Design	6
2.5 IG Pretreatments.....	7
2.6 Statistical Methods	8
3.0 RESULTS.....	9
4.0 DISCUSSION	15
5.0 CONCLUSIONS	16
6.0 ARCHIVES	17
7.0 ACKNOWLEDGEMENTS	17
8.0 REFERENCES.....	18

LIST OF APPENDICES

- APPENDIX A: STUDY PROTOCOL MREF 130 (AND AMENDMENTS) – PROTECTION OF GUINEA PIGS BY PASSIVE IMMUNIZATION WITH POOLED HUMAN IMMUNE GLOBULIN OBTAINED FOLLOWING VACCINATION
- APPENDIX B: STUDY DEVIATIONS
- APPENDIX C: STANDARD OPERATING PROCEDURE (SOP) FOR THE DETECTION AND QUANTITATION OF BOTULINUM TOXINS AND ANTITOXINS (ANTIBODIES)
- APPENDIX D: STATISTICAL REPORTS FOR MORTALITY DATA
- APPENDIX E: STATISTICAL REPORTS FOR CLINICAL DATA
- APPENDIX F: CHEMISTRY REPORTS FOR MONOMERIC IgG OF THE PURIFIED, LYOPHILIZED IMMUNOGLOBULINS
- APPENDIX G: QUANTITATIVE ANALYSES OF PURIFIED IMMUNOGLOBULINS
- APPENDIX H: SCIMEDX CORP REPORTS
- APPENDIX I: LIST OF PLASMA SAMPLES SENT TO SCIMEDX CORP. FOR PURIFICATION AND LYOPHILIZATION
- APPENDIX J: IMMUNE GLOBULIN NAC DETERMINATION (U/ML)
- APPENDIX K: VALIDATION OF ANTITOXIN STANDARDS FOR SEROTYPES A, C, AND D.

LIST OF TABLES

	<u>Page</u>
Table 1. Mean Neutralizing Antibody Concentrations (NAC) in Guinea Pigs 24 Hours after Administration of 10 U/kg of IG Preparations	9
Table 2. Estimated LD ₅₀ s and Fieller's 95 Percent Confidence Intervals For Each IG Type	10
Table 3. Estimated Protective Ratios of LD ₅₀ - IG: LD ₅₀ -CIG for Serotype A.....	10
Table 4. Estimated L+/10, L+/33 and L+/100 Test Dosages and Avidity Measurements Using PBiGA, PBiGB, BBIG, PBiGAB, and BIG for Serotype A.....	13

ABBREVIATIONS

BIG – Botulism Immune Globulin	NAC – Neutralizing Antibody Concentration
BBIG – Booster Botulinum Immune Globulin	PBS – Phosphate-Buffered Saline
CIG- Control Immune Globulin	PBT – Pentavalent Botulinum Toxoid
CDC – Centers for Disease Control and Prevention	PBIG – Primary Botulinum Immune Globulin
DE – Droopy Eyelids	PBIGA - Primary Botulinum Immune Globulin, Lot A
ED ₅₀ – Median Effective Dose	PBIGB - Primary Botulinum Immune Globulin, Lot B
FDA – Food and Drug Administration	PBIGAB – Primary Botulinum Immune Globulin – Equal volumes of PBIGA and PBIGB combined
GPB – Gelatin Phosphate Buffer	RF – Ruffled Fur
IG – Immune Globulin	S – Salivation
IM – Intramuscular	SOP – Standard Operating Procedures
IND – Investigational New Drug	TP – Total Paralysis
IP – Intraperitoneal	TSI – The Salk Institute
IU– International Units	U – Unit
Kg – Kilogram	USAMRIID – U.S. Army Medical Research Institute of Infectious Diseases
L – Lacrimation	VIG – Vaccinia Immune Globulin
LB – Labored Breathing	WHO – World Health Organization
LC _{t50} – Median Lethal Concentration	WL – Weak Limbs
LD ₅₀ – Median Lethal Dose	
LOQ – Limit of Quantitation	
LP – Local Paralysis	
MIPLD ₅₀ – Mouse Intraperitoneal LD ₅₀	
mL – Milliliter	
MREF – Medical Research and Evaluation Facility	

LIST OF FIGURES

	<u>Page</u>
Figure 1. Predicted Percent Lethality Plotted Against Serotype A for Five IG Types. (For Comparison, the Two Serotype A Groups from Task 97-52 are also Plotted).....	12
Figure 2. Neutralizing Potency at ED ₅₀ Relative to L+/10 Dilution using PBIGA, PBIGB, BBIG, PBIGAB and BIG for Serotype A.....	14

FINAL REPORT

**Contract No. DAMD17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program**

on

TASK 97-53

**Protection of Guinea Pigs by Passive Immunization with Human Botulinum Immune
Globulin Obtained Post Primary Series and Post Six-Month Booster Immunization**

to

JOINT PROGRAM OFFICE FOR BIOLOGICAL DEFENSE

January 2001

by

**Dr. Robert E. Hunt
Ms. Rebekah A. Starner
Ms. Michelle L. Clagett
Ms. Nancy A. Niemuth
Ms. M. Claire Matthews**

**BATTELLE
Medical Research and Evaluation Facility (MREF)
Bldg. JM-1
State Route 142
Plain City Georgesville Road
West Jefferson, Ohio 43162**

Approved for public release; distribution is unlimited.

FINAL REPORT

on

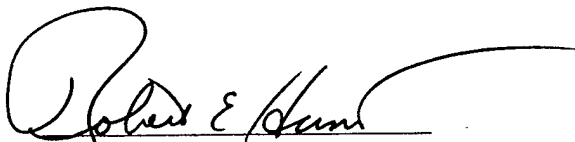
Task 97-53

Protection of Guinea Pigs by Passive Immunization with Human Botulinum Immune Globulin
Obtained Post Primary Series and Post Six-Month Booster Immunization

to

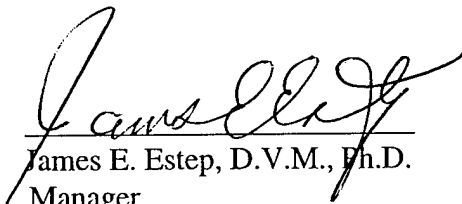
JOINT PROGRAM OFFICE FOR BIOLOGICAL DEFENSE

January 2001



Robert E. Hunt, D.V.M.
Study Director

9 Jan 2001
Date

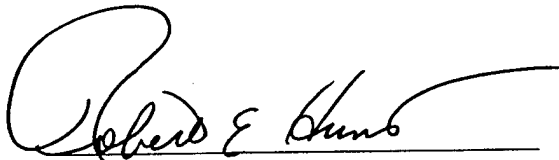


James E. Estep, D.V.M., Ph.D.
Manager
Medical Research and Evaluation Facility

01-09-01
Date

GLP COMPLIANCE STATEMENT

Procedures were performed at Battelle's Medical Research and Evaluation Facility (MREF) in compliance with the Food and Drug Administration's Good Laboratory Practice regulations (21 CFR Part 58). The study was conducted according to the study protocol, as amended, and Battelle's standard operation procedures. Deviations from the protocol or standard operating procedures are documented. The data presented accurately reflect the results of the study.



Robert E. Hunt, D.V.M.
Study Director

9 Jan 2001
Date

EXECUTIVE SUMMARY

Studies (MREF Tasks 95-39, 96-45, 97-51, and 97-52) have previously demonstrated a high degree of correlation between circulating neutralizing antibody titers and protection against high doses of all botulinum toxin serotypes in the guinea pig model. Neutralizing antibodies have been proposed to the FDA as a serological marker for human protection since efficacy for this vaccine cannot be directly demonstrated in traditional human clinical trials. Task 97-53 establishes the level of passive protection conferred in the guinea pig model by pretreatment with botulism immune globulin (BIG) isolated and purified from human volunteers immunized with one-of-two different Pentavalent Botulinum Toxoid Adsorbed (PBT) vaccine lots (arbitrarily designated A or B).

Botulinum immune globulin prepared from volunteers immunized with either vaccine lot and with both a primary vaccination series (designated PBIGA or PBIGB) and a six-month booster immunization (designated BBIG which was a pooling of both vaccine lots prior to purification and lyophilization), as well as a combined PBIG preparation of equal volumes of the reconstituted PBIGA and PBIGB (designated PBIGAB) were compared against the lethal effects of botulinum serotype A in guinea pigs, as measured by a protective ratio (median lethal dose-protected : median lethal dose-unprotected). Guinea pigs were treated to attain a neutralizing antibody concentration (NAC) of approximately 0.06 U/mL. Control animals received an identical amount of immune globulin from a non-immunized control population (designated CIG). All CIG treatments were normalized to the total protein content of the PBIGA plasma since it contained the highest protein level. Twenty-four hours following pretreatment with the immune globulin, the guinea pigs were challenged with various doses of botulinum serotype A toxin by the intramuscular route (IM) and the protective ratios calculated. The median lethal dose (LD₅₀) was determined using a stage-wise approach with up to four iterations. Probit analyses were based on fourteen-day survival data following toxin challenge. Toxin dosages were verified by an *in vivo* mouse potency assay on each day of experimentation and expressed in terms of mouse intraperitoneal median lethal dose (MIPLD₅₀) units. At the time of toxin

challenge, serum samples were obtained from randomly selected immune globulin pretreated but unchallenged guinea pigs and measured by the mouse neutralization assay to determine the circulating antibody levels. Mean NACs in guinea pig serum samples 24 hr after PBIGA, PBIGB, PBIGAB, and BBIG administration were 0.058, 0.057, 0.057, and 0.082 U/mL serum, respectively.

Antibody avidity was evaluated for all current IG preparations and a human botulism immune globulin preparation (designated BIG) against botulinum serotype A. BIG (lot IVBG-1B, Univ. of MN, IND 1332) was produced in volunteers who had received multiple immunizations of PBT (Michigan Department of Public Health) vaccine, but not the same vaccine lots.

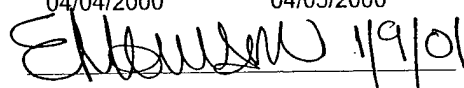
There were no significant differences between the protective ratios of PBIGA, PBIGB, PBIGAB, or the pooled BBIG, and all conferred substantial protection with median lethal dose estimates of 84,000, 150,000, 103,000, and 135,000 MIPLD₅₀ units per injection, respectively. The protective ratios (LD₅₀IG:LD₅₀CIG) were 7,400, 13,200, 9,000, and 11,800, respectively, against serotype A. The median lethal dose estimate for CIG-pretreated groups was 11.5 MIPLD₅₀ units, showing no significant protective value to the guinea pigs. When the protective ratios of these current IG preparations were compared against historical data (MREF Task 97-52) generated using BIG, the protective ratios for all preparations were significantly higher (approximately 15-25 times higher) than for the BIG. The IG preparations with the lowest avidity were PBIGA and PBIGB. The neutralizing antibody avidity appears to increase as a function of the number of immunizations or by pooling of the sera immunized with different PBT lots. In summary, NAC correlates with protection; however, it is not predictive of the magnitude of protection. For example, a 10 U/kg BIG dose was administered to guinea pigs to attain a serum NAC of approximately 0.06 U/mL and demonstrated a protective ratio of 480. However, the same dose of IG prepared from PBT lots A and B was administered to guinea pigs to attain a serum NAC of approximately 0.06 U/mL and demonstrated a protective ratio ranging from 7,400-13,200.

Quality Assurance Statement

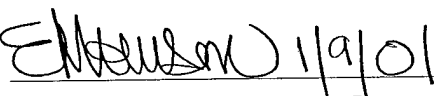
Study Number: G155553A

This Study was inspected by the Quality Assurance Unit and reports were submitted to the Study Director and management as follows:

<i>Phase Inspected</i>	<i>Inspection Date</i>	<i>Date Reported To Study Director</i>	<i>Date of Report to Management</i>
Draft protocol	08/22/1997	08/29/1997	11/05/1997
Protocol Review	07/22/1998	07/22/1998	07/24/1998
Validation of assays & standards	08/11/1998	08/13/1998	08/17/1998
Validation of assays & standards	08/13/1998	08/13/1998	08/17/1998
Preparation of dilutions	12/16/1998	12/16/1998	12/21/1998
Animal observation (post dose)	12/16/1998	12/16/1998	12/21/1998
Weighing of mice and animal husbandry	12/16/1998	12/16/1998	12/21/1998
Protocol amendment 1	06/14/1999	06/15/1999	06/16/1999
Packing plasma samples for shipment	07/06/1999	07/06/1999	10/29/1999
Plasma inventory records	07/06/1999	07/06/1999	10/29/1999
Protocol amendment 2	10/04/1999	10/05/1999	10/05/1999
Packing plasma samples for shipment	10/26/1999	10/28/1999	10/29/1999
Plasma inventory records	10/28/1999	10/28/1999	10/29/1999
Toxin stability and optimization of antitoxin	11/03/1999	11/04/1999	11/15/1999
Validation of assays	12/29/1999	12/30/1999	01/04/2000
Preparation of dilutions	01/17/2000	01/18/2000	01/19/2000
Dosing of mice	01/17/2000	01/18/2000	01/19/2000
Animal observation (post dose)	01/18/2000	01/18/2000	01/21/2000
Animal husbandry	01/18/2000	01/18/2000	01/21/2000
Protocol Amendment 3	02/17/2000	02/17/2000	03/07/2000
Reconstitution of purified plasma and transfer	02/23/2000	02/23/2000	02/24/2000
Avidity- Dilutions, animal weighing and dosing	03/06/2000	03/07/2000	03/09/2000
Animal obs post dose and husbandry	03/08/2000	03/08/2000	03/09/2000
Protocol amendment 4	03/10/2000	03/10/2000	12/28/2000
Guinea pig receipt and tatoo	03/23/2000	03/23/2000	04/11/2000
Reconstitution of purified plasma	03/28/2000	03/28/2000	04/05/2000
Weighing of guinea pigs	03/28/2000	03/28/2000	04/05/2000
Drawing up syringes and immunization	03/28/2000	03/28/2000	04/05/2000
Drawing up syringes and dosing	03/29/2000	03/29/2000	04/05/2000
Animal husbandry, clinical obs and bleeding	03/29/2000	03/29/2000	04/05/2000
Guinea pig bleeding and euthanasia	03/29/2000	03/30/2000	04/05/2000
Randomization	04/04/2000	04/04/2000	04/05/2000
Receipt, inventory and storage of test material	04/04/2000	04/04/2000	04/05/2000


Quality Assurance Unit *Date*

<i>Phase Inspected</i>	<i>Inspection Date</i>	<i>Date Reported To Study Director</i>	<i>Date of Report to Management</i>
Study binder 1 and SAS binder 1	06/14/2000	06/15/2000	07/24/2000
Study binder 2	08/18/2000	08/18/2000	10/10/2000
Study binder 3 and guinea pig listing	08/23/2000	08/24/2000	11/01/2000
Guinea pig listing	10/31/2000	10/31/2000	12/12/2000
Chemistry Reports	11/14/2000	11/14/2000	12/05/2000
Plasma record book and binders 2 & 3	11/20/2000	11/20/2000	12/29/2000
Draft Final Report	11/20/2000	11/20/2000	12/29/2000
Test/control article logbook	12/12/2000	12/12/2000	01/09/2001
Facility records (binder 2)	12/18/2000	12/19/2000	01/09/2001
Facility records (binder 1)	12/18/2000	12/19/2000	01/09/2001
Administrative and statistics binders	12/28/2000	12/28/2000	01/09/2001
Draft Final Report	12/28/2000	12/28/2000	01/09/2001
Final Report	01/09/2001	01/09/2001	01/09/2001


 Quality Assurance Unit Date

TASK 97-53**Protection of Guinea Pigs by Passive Immunization with Human Botulinum Immune Globulin Obtained Post Primary Series and Post Six-Month Booster Immunization****1.0 INTRODUCTION**

Botulinum Toxoid Adsorbed Pentavalent (A-E) vaccine, also called Pentavalent Botulinum Toxoid (PBT) and Pentavalent (ABCDE) Botulinum Toxoid, Aluminum Phosphate Adsorbed, is produced by BioPort, Inc., formerly the Michigan Department of Public Health (MDPH). PBT vaccine was designed to protect military personnel from battlefield exposures to botulinum toxin serotypes A-E. This product has also been used to protect laboratory workers at risk for botulinum due to contact with toxins produced by *Clostridium botulinum*. The CDC recommended immunization schedule for the PBT vaccine is 0, 2, and 12 weeks, and a one-year booster immunization. Subsequent booster immunizations are scheduled at 2-year intervals if needed. The PBT vaccine is in the FDA Investigational New Drug (IND) category. To attain licensure for a product, the Food and Drug Administration (FDA) usually requires that efficacy be demonstrated in humans. Typically, vaccine efficacy is demonstrated during clinical trials with at-risk populations. However, the highly toxic nature of the botulinum toxins precludes direct challenge studies in humans and the extremely low incidence of naturally occurring botulism prevents field studies of naturally occurring disease. As an alternative, the measurement of neutralizing botulinum antibodies has been proposed to serve as a serological correlate of protection.

Over the last several decades, the neutralization assay (Cardella, 1964) has been used by the Centers for Disease Control and Prevention (CDC), The Salk Institute (TSI), and the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) to measure neutralizing antibodies in serum samples from volunteers vaccinated with PBT (Ellis, 1982; Seigel, 1988). A series of studies have been conducted at Battelle's Medical Research and Evaluation Facility (MREF) to establish a relationship between neutralizing antibody levels in serum, as measured by this assay, and the magnitude of protection from lethal challenges with botulinum toxins in the guinea pig model. In a previous study (Gelzleichter et al., 1998a), the minimum-circulating antibody levels in serum that conferred protection against lethal inhalation challenges

(25 LC₅₀) with botulinum toxins A-E were determined. Antibodies isolated from volunteers vaccinated with PBT were passively administered to guinea pigs by intraperitoneal (IP) injection to attain desired circulating antibody levels. MREF Tasks 97-52 and 97-53 were designed to measure the magnitude of protection provided by a target circulating antibody level of 0.06 U/mL, with human antibodies isolated at different time points in the immunization process. In both of these studies, guinea pigs were administered human immune globulin (IG) at a dosage necessary to attain approximately 0.06 U/mL in circulating serum. Twenty-four hours after this pretreatment, animals were challenged by intramuscular (IM) injection with varying doses of botulinum toxins to determine protective ratios (median lethal dose-protected : median lethal dose-unprotected).

The Botulism Immune Globulin (BIG) used in Task 97-52 experiments was originally intended as a therapeutic (BB-IND 1332) and was collected from volunteers with very mature immune responses to PBT (e.g., primary vaccination plus numerous booster vaccinations). In contrast, the immune globulin used in Task 97-53 was obtained approximately four weeks after a primary vaccination series (day 113) or approximately four weeks after a six-month booster vaccination (day 211). Task 53 was performed under MREF Protocol 130 entitled, "Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin Obtained Following Vaccination" (Appendix A). The experimental objectives were to a) determine the level of protection provided by these immune globulin preparations against the lethal effects of botulinum toxins, as measured by protective ratios, b) identify if significant differences exist in the level of protection provided by antibody collected on day 113 or day 211 post vaccination, and c) compare differences in avidity between the BIG, PBIG and BBIG preparations. The combined results of Tasks 97-52 and 97-53 can be used to evaluate the relative efficacy of antibodies isolated at different times in the vaccination process to evaluate the value of NAC as a serological correlate of protection.

2.0 MATERIALS AND METHODS

2.1 Animals

Male Hartley CrI:(HA)BR guinea pigs weighing between approximately 375 and 475 grams (approximately 28-35 days old) served as the test system and were obtained from Charles River Breeding Laboratories Inc. (Portage, MI or Stonebridge, NY). Male CrI:CD-1 (ICR)BR mice (approximately 25-35 days old) were obtained from Charles River Laboratories (Portage, MI or St. Constant, Quebec). For toxin potency assays, mice weights ranged between 18.0 and 22.0 grams and for the neutralization assays weights ranged between 17.0 and 23.0 grams. Guinea pigs were prescreened for pneumonia and Sendai virus. Mice were purchased sero-negative for Theiler's encephalomyelitis, minute virus, reovirus 3, pneumonia virus, polyoma, ectromelia, Sendai viruses, mouse hepatitis virus, lymphocytic choriomeningitis, mouse adenovirus, K virus, and enzootic diarrhea as tested by Charles River Labs. During and following quarantine, animals were housed in stainless steel cages in stainless steel racks equipped with automated watering systems. Water is analyzed for chemical impurities and potability annually. Guinea pigs and mice received Certified Guinea Pig Chow[®] and PMI Certified Rodent Chow[®], respectively. Both feed and water were provided *ad libitum*. No contaminants that would interfere with the results of this study were known to be present in the food or water. Guinea pigs were tattooed on the ear to retain positive identification. The endpoint for the mouse assays was percent lethality per treatment group so mice were identified by group only. Once mice were treated and assigned to home cages, living mice were not moved to different caging during the 96 hour observation period. Procedures to avoid mixing of mice were carried out as per Appendix C. All animal rooms were placed on a light/dark cycle of approximately 12 hr each per day. Guinea pigs were randomly distributed to dosage groups using a computer-generated randomization algorithm. All animals were quarantined for a minimum of four days prior to use on study. A total of 252 guinea pigs and approximately 20,908 mice were used on this study.

2.2 Antibody Preparations

The test and control article(s) for this study consisted of human plasma collected from volunteers on a study entitled "Phase I/II Evaluation of Safety and Immunogenicity of Pentavalent Botulinum Toxoid (A-E) Administered to Health Volunteers", (IND number 3723).

The plasma was received frozen from MDS Harris Laboratories (Phoenix, AZ), as part of MREF Task 161. Samples received were labeled with a date, quantity, bleed, donor, and bar-coded sample identification number. The plasma was stored at approximately -20°C until shipped to SciMedX Corporation (Denville, NJ) for purification and lyophilization.

The frozen plasma was collated into lots of approximately 15 liters according to the vaccine lot (designated A or B per list from MDS Harris) that was used to immunize the volunteer and the corresponding plasmaphoresis study date (day 113 or 211). The actual vaccine lot (PBT003 or PBT004) used to immunize the volunteers was not known to Task 97-53 study personnel and the study was conducted in a blinded fashion to preclude any perceived bias. The frozen plasma was shipped on dry ice to SciMedX Corp (Denville, NJ), for purification and lyophilization. Each 15-liter plasma batch was purified by Cohn (method-6) – Oncley (method-9) cold ethanol fractionation, given a unique lot number, and tested for endotoxin levels prior to being returned to the MREF for chemical assessment and *in vivo* testing. Plasma collected on day 113 or day 211 following vaccination was designated primary botulinum immune globulin (PBIG) and booster botulinum immune globulin (BBIG), respectively. Two lots of control plasma (CIG) from non-immunized volunteers (day 0) were prepared as the control article; however, only one lot (D1060) was used in the course of the study. The six-month booster plasma collected on day 211 was available in a very limited quantity (volunteers dropped out of the clinical trial prior to plasmaphoresis), therefore the plasma collected from Lot A volunteers and Lot B volunteers was commingled at SciMedX Corp prior to purification and lyophilization into a single lot in order to provide an adequate quantity of purified BBIG for these studies. Equal volumes of reconstituted PBIGA and PBIGB were mixed during dilution preparation at the MREF to generate a single lot (designated PBIGAB) for the comparison of BBIG against PBIG. Thus, the control article was designated as CIG (day 0), and the test articles were designated as PBIGA or PBIGB (day 113), PBIGAB (day 113), and BBIG (day 211). These vials of lyophilized and purified plasma were stored at approximately 4°C and reconstituted in 2-mL of

sterile water for injection immediately prior to use. Any remaining reconstituted product was destroyed at the end of each day. Since the material was used within one-hour of re-constitution, long-term stability of the test article was not considered to be an issue and stability was not tested.

All lyophilized and purified lots of plasma were greater than 90 percent monomeric IgG by fast protein liquid chromatography (FPLC) method (Appendix F). Reconstituted purified plasma samples were submitted by courier on wet ice to LabCorp, Inc. (Dublin, OH) for laboratory quantitative analysis of total protein, total IgG, IgA, IgM, and IgG subclasses 1,2,3, and 4. See Appendix G for a characterization of immune globulin preparations.

Lyophilized vials stored under controlled refrigerated conditions at the MREF were reconstituted and assayed by the mouse neutralization assay to determine relative recovery of neutralizing antibody against serotypes A-E. World Health Organization (WHO) international standards issued in 1963 and 1985 were used as standards for the neutralization assay for serotypes B and E, respectively. International standards were not available for serotypes A, C, and D, thus, PerImmune Inc. standards (1998 issue; Appendix K) were used for these serotypes.

Once an approximate titer was established, two standard curves were performed and averaged to determine the final titer value (Appendix J). Based upon the average NAC attained in the preliminary guinea pig studies, it was determined that the available quantity of any one specific test article would only support testing against a single serotype of botulinum toxin. Botulinum serotype A was selected by the sponsor as the challenge toxin and the study design was amended to accommodate testing of a single serotype.

2.3 Toxin Preparations

Partially purified botulinum toxins A, B, C, D, and E were produced at the University of Wisconsin in the laboratories of Dr. Eric Johnson and previously shipped to Battelle as ammonium sulfate precipitates under refrigerated conditions. Prior to use on study, toxins were reconstituted in phosphate-buffered saline (PBS, pH 6.2), dispensed in small vials and placed in storage at approximately -70 degrees Celsius. All toxin samples were used immediately after thawing and were not refrozen or reused. The manufacturer's lot numbers were A011995,

B011995, C012495, D022505, and E011295 for serotypes A-E, respectively. Botulinum toxin serotype E was trypsin-treated prior to administration (Method provided in Appendix C).

2.4 Experimental Design

Protective Ratio

To characterize the level of protection conferred by various IG pretreatments, protective ratios (defined as $LD_{50}\text{-protected} : LD_{50}\text{-unprotected}$) were determined for botulinum toxin serotype A. The $LD_{50}\text{-unprotected}$ was defined as the estimated dosage of toxin at which 50 percent of CIG-treated animals died within fourteen days. Likewise, the $LD_{50}\text{-protected}$ was defined as the estimated dosage of toxin at which 50 percent of IG-treated animals survived within fourteen days. LD_{50} values were determined using a stage-wise approach (Feder et al., 1991). Using this approach, small numbers of animals were pretreated in each of several sequential iterations. For the first iteration, six IG-pretreated guinea pigs received approximately 6, 25, 100, 400, 1600, or 6400 times the guinea pig LD_{50} of 4.3 MIPLD₅₀ per animal, as estimated from a previous task (Gelzleichter et al., 1998b). Six CIG-treated guinea pigs received doses logarithmically spaced around the LD_{50} estimate. As each of the iterations was completed, doses for the next iteration were calculated based on all the available information and selected to fall in close proximity with the anticipated LD_{50} . For the remaining iterations, typically three to four guinea pigs per dose were used to compile dose-response data. Twelve (12) IG- and 12 CIG-pretreated guinea pigs were used for each of iterations two and three. If after three iterations the desired level of statistical precision of the LD_{50} estimate was not yet reached, a fourth and final iteration was conducted with ten guinea pigs for each IG preparation.

Avidity

Avidity was characterized for PBIGA, PBIGB, PBIGAB, BBIG, and BIG by measuring relative neutralizing capability at three widely varying antibody concentrations. Efficiency ratios were determined using the same procedures as reported by Gelzleichter [MREF Task 39 Final Report].

In brief, this test was performed using the following fixed antibody concentrations for each IG preparation: 0.1, 0.03 and 0.01 units of IG preparation per injection in gelatin phosphate buffer

(GPB). Each IG preparation, at each antibody concentration listed above, was mixed with serial dilutions of serotype A botulinum toxin and injected IP into mice in 0.5 mL volumes. The amount of toxin per injection that was 50 percent neutralized was determined by probit analysis and is referred to as L+/10, L+/33, and L+/100 test levels where samples were titrated against 0.1, 0.03 and 0.01 units of antitoxin per injection, respectively. A broad based range finding curve was constructed using 2 mice per group (ten groups total) to determine an approximation of the LD₅₀. Once an approximate range was established, the avidity was determined with a single curve using at a minimum, five mice per group. A potency experiment was performed concurrently with the avidity experiments for use in the toxin dose calculation (MIPLD₅₀ units per injection).

2.5 IG Pretreatments

To attain a serum NAC of approximately 0.06 U/mL at 24 hr after administration, guinea pigs were given a 10 U/kg dose by IP injection. This was performed for each IG preparation. Each iteration of test animals was pretreated on the same day. This dose (10 U/kg) was shown to produce close to the desired NAC for all five serotypes (Gelzleichter et al., 1998a). Control guinea pigs received an equivalent dose of CIG normalized to PBIGA protein content. All iterations included unchallenged guinea pigs for serological monitoring to verify serum NAC. At 24±1 hours after IG administration, four "sentinel" guinea pigs were randomly selected from each pretreatment group per iteration to determine serum NAC using a mouse bioassay (Cardella, 1964). A detailed description of this assay is provided in Appendix C. At the time of serum sampling, the remaining guinea pigs were challenged with varying concentrations of botulinum serotype A toxin by IM administration. Toxin preparations were diluted in GPB (30 mM phosphate, 0.2 percent gelatin, pH 6.2) and administered to guinea pigs by injection into the quadriceps muscle group. On each day of toxin challenge, potency was determined using an *in vivo* mouse lethality assay (see Appendix C) and expressed in terms of mouse intraperitoneal LD₅₀ (MIPLD₅₀) units. Clinical observations were recorded twice daily for guinea pigs during a 14-day postchallenge observation period. The following categories were used for botulism-related clinical signs: ruffled fur (RF), labored breathing (LB), droopy eyelids (DE), weakness in limbs (WL), total paralysis (TP), salivation (S), lacrimation (L) and local paralysis (LP). Local

paralysis entails paralysis of the hind limb that received the botulinum toxin injection. Nonbotulism-related signs were recorded independent of these categories.

2.6 Statistical Methods

The mouse neutralization assay was used to determine the serum NAC achieved in the sentinel animals passively immunized with the various IG types. Descriptive statistics (means, standard deviations, and 95 percent confidence bounds for the means) were computed for the NACs from sentinel animals for each IG-treated group, including the BIG group from Task 97-52. A one-way analysis of variance (ANOVA) was used to test the significance of IG type effects upon the NACs, and a Tukey's Multiple Comparison Test was used to rank and group the IG types according to their mean NACs.

Probit dose-response models were fitted to the guinea pig lethality data as a function of the GP doses for each IG preparation. Estimated parameters of the probit dose-response models were used to calculate the LD₅₀ for each IG type, together with 95 percent confidence intervals using Fieller's method (Finney, 1971). LD₅₀ ratios and protective ratios with 95 percent confidence intervals were calculated for various IG combinations, including ratios relative to the LD₅₀s from Task 97-52. Probit dose-response models were fitted using the SAS (V8) PROBIT procedure. Doses were expressed as MIPLD₅₀ units per animal and were calculated as:

$$\text{GP dose} = \text{injection volume (mL)} * \text{dose dilution} * \text{toxin potency},$$

where toxin potency is the MIPLD₅₀ per mL established by a mouse potency assay run on the challenge day. A more detailed description of the statistical analysis performed on the guinea pig lethality data can be found in Appendix D.

A report outlining the statistical analysis performed on the guinea pig clinical signs is located in Appendix E.

3.0 RESULTS

A subset of guinea pigs were administered either 10 U/kg IG or an equivalent amount of CIG (normalized to PBIGA protein content) and their sera assayed 24 hours later to determine serum NAC. The target NAC was 0.06 U/mL. Table 1 presents descriptive statistics for NAC in five groups of IG-treated animals from Task 97-52 and Task 97-53, footnoted with the results from the Tukey comparisons.

Table 1. Mean Neutralizing Antibody Concentrations (NAC) in Guinea Pigs 24 Hr after Administration of 10 U/kg of IG Preparation

IG Type	No. of Experiments	NAC, Mean (U/mL) (*)	95% Confidence Interval Mean NAC
PBIGAB	16	0.057 ^x	(0.049, 0.064)
PBIGB	11	0.057 ^x	(0.045, 0.069)
PBIGA	15	0.058 ^x	(0.048, 0.068)
Task 52, BIG	16	0.073 ^{x,y}	(0.061, 0.085)
BBIG	12	0.082 ^y	(0.070, 0.094)

(*) x,y = Letters indicating groupings of similar IG types according to Tukey comparisons. Any means not followed by the same letter are significantly different at the p=0.05 level.

The Tukey's Multiple Comparison Test showed that mean NACs were not statistically different among the primary series injection groups (PBIGA, PBIGB, and PBIGAB). The average of these three group means was approximately 0.057 U/mL. The mean NAC of 0.082 U/mL in the booster injection group was significantly greater than the mean NACs in the three primary injection groups. By comparison, the mean NAC level in the Task 97-52 BIG animals was intermediate in value (approximately 0.073 U/mL) and was not significantly different from any of the Task 97-53 groups. All of the NAC values for the CIG-treated animals (16/16) were below the Limit of Quantitation (LOQ) values of approximately 0.02. Only one of the 72 IG-treated sentinel guinea pigs had a NAC below the LOQ.

Protective Ratio

Estimated LD₅₀ values, 95 percent confidence intervals, and probit slopes for CIG and IG-treatment groups are provided in Table 2 and estimated protective ratios (LD₅₀IG : LD₅₀CIG) are provided in Table 3.

Table 2. Estimated LD₅₀s And Fieller's 95 Percent Confidence Intervals For Each IG Type

IG Type Used with Serotype A	N	Probit Slope (*)	MIPLD ₅₀ Units Per Animal	Fieller's 95 Percent Confidence Interval for LD ₅₀
PBIGA	40	1.7	84,000	(6,300, 233,000)
PBIGB	30	5.7	150,000	(103,000, 240,000)
PBIGAB	40	1.8	103,000	(24,000, 206,000)
BBIG	30	5.6	135,000	(91,000, 209,000)
CIG	40	4.1	11.4	(8.50, 19.9)
Task 97-52, BIG	40	1.4	1,900	(460, 3800)
Task 97-52, VIG	40	2.6	4.0	(1.70, 6.00)

(*) All probit slopes were significantly greater than zero ($p \leq 0.05$).

Table 3. Estimated Protective Ratios of LD₅₀-IG : LD₅₀-CIG for Serotype A

IG	Protective Ratio (LD ₅₀ -IG : LD ₅₀ -CIG)	95% Confidence Interval for Protective Ratio	IG/CIG Ratio Significantly Different Than 1
PBIGA	7,400	(3,800, 14,200)	Yes, <0.0001
PBIGB	13,200	(8,700, 19,900)	Yes, <0.0001
PBIGAB	9,000	(4,600, 17,800)	Yes, <0.0001
BBIG	11,800	(7,800, 17,900)	Yes, <0.0001
BIG*	480	(190, 1,200)	Yes, <0.0001

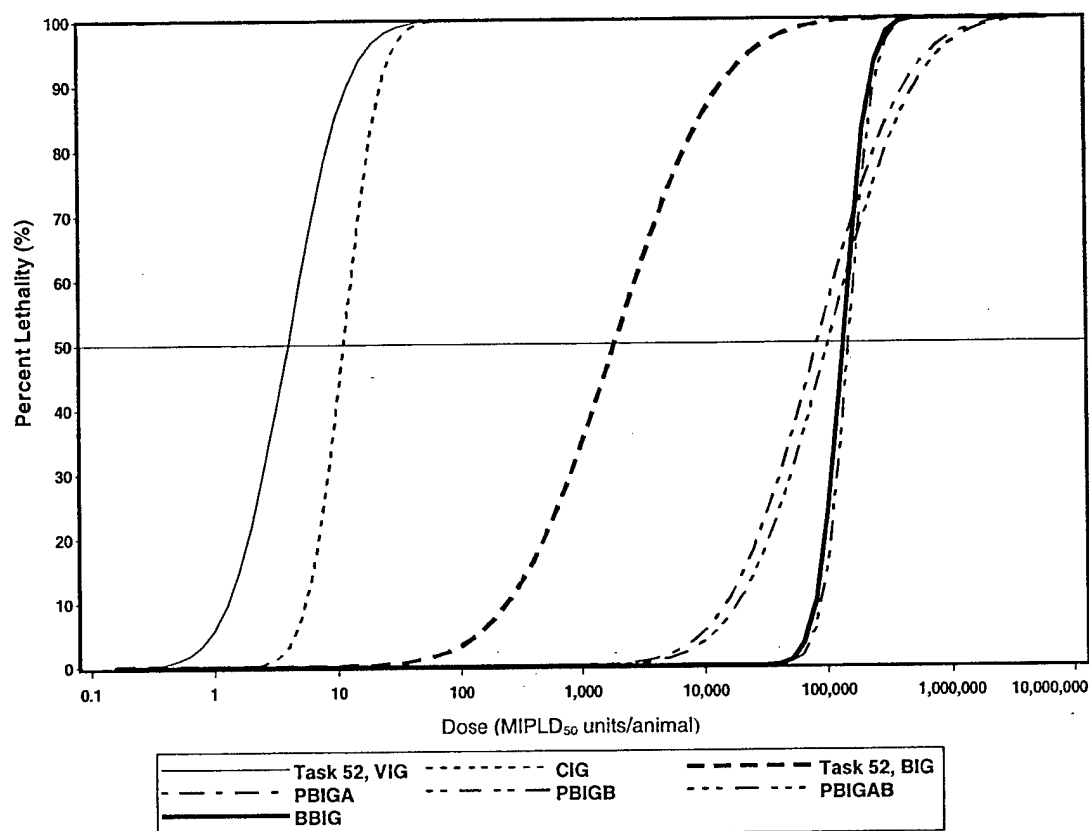
* Historical data from Task 97-52

LD₅₀ estimates were significantly higher for all IG-pretreated guinea pigs than for CIG-pretreated guinea pigs ($p < 0.0001$ in all cases). The LD₅₀ values were not statistically different

among the PBIGA, PBIGB, PBIGAB, and BBIG groups. The geometric mean of the four LD_{50} s was 115,000 MIPLD₅₀ units per animal. Even though the LD_{50} from the Task 53 CIG control group was significantly greater than the Task 97-52 VIG control group, both demonstrated a lack of protection compared to the IG preparations. The LD_{50} s for the PBIG- and BBIG-treated animals were significantly higher than the LD_{50} from the BIG treated animals in Task 97-52. Wide confidence intervals (noted in Table 2 for PBIGA) may be attributed to two of the four-mouse potency experiments having one toxin concentration group with a lethality rate that was considered an "outlier". Since no errors in the procedures were noted, the groups were not excluded from the potency experiments. To ensure that the same patterns of similarities and differences among IG types were evident if the potency experiments were adjusted, statistical analyses was performed with and without these "outlier" groups (Appendix D). The LD_{50} estimates were fairly consistent between the analyses ("unadjusted" vs "adjusted") however the confidence intervals were narrowed using the "adjusted" potencies. For both sets of analyses, the geometric mean LD_{50} taken across the PBIGA, PBIGB, PBIGAB, and BBIG groups was more than 10,000 times greater than the corresponding LD_{50} for the corresponding CIG or VIG control group.

Figure 1 displays the dose-lethality curves predicted by the probit analysis for the five IG preparations from Task 97-53 and two IG preparations from 97-52. The same patterns of group similarities and differences can be seen. The LD_{50} s for the seven IG preparations correspond to the guinea pig doses where each curve crosses the horizontal reference line at 0.5 proportion lethality.

Figure 1. Predicted Percent Lethality Plotted Against Serotype A for Five IG Types. For Comparison, the Two Serotype A Groups from Task 97-52 are also Plotted.



A summary of total observed clinical signs is provided in Appendix E. Statistically significant differences did occur between the IG pretreatment groups and CIG in time to onset and duration of clinical signs. However, these differences were less than a day and were not considered clinically relevant. In general, types, percent incidence, and temporal progression of botulism-related clinical signs were similar for both CIG and IG pretreatment groups.

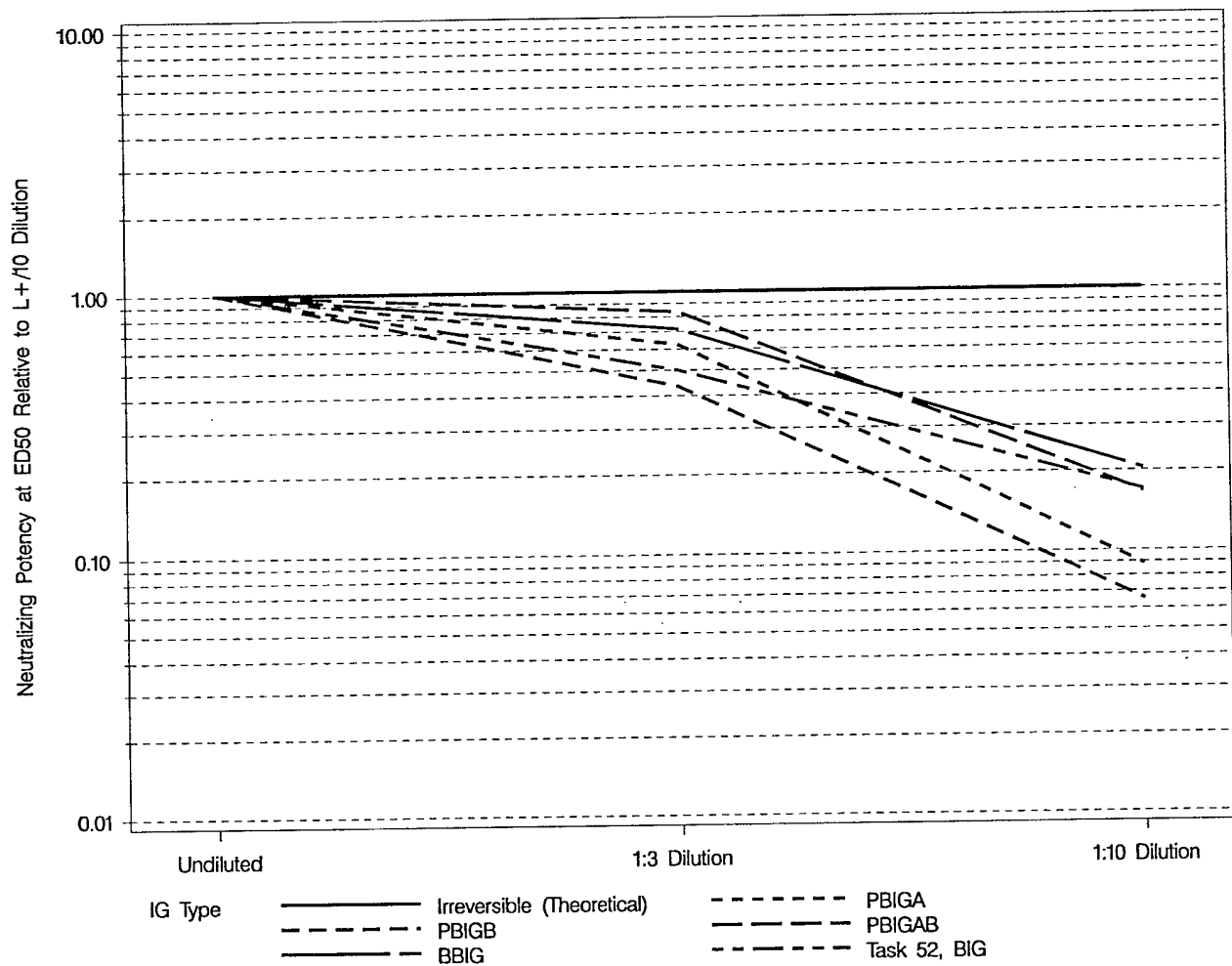
Avidity

Results of the avidity determinations are displayed in Table 4 and Figure 2.

Table 4: Estimated L+/10, L+/33 and L+/100 Test Dosages and Avidity Measurements Using PBIGA, PBIGB, BBIG, PBIGAB and BIG for Serotype A

	Level of Test	Reference Assay Number	Number of MIPLD ₅₀ Units Per Injection at Test Level	Test Ratio	Avidity Measurements Ratio
PBIGA	L+/10	11007	5,300	$[L+ /10] / [3.3 *L +/33]$	1.6
	L+/33	11008	1,010	$[L+ /33] / [3 *L +/100]$	7.2
	L+/100	11009	47	$[L+ /10] / [10 *L +/100]$	11
PBIGB	L+/10	21010	8,200	$[L+ /10] / [3.3 *L +/33]$	2.3
	L+/33	21008	1,100	$[L+ /33] / [3 *L +/100]$	6.8
	L+/100	21009	54	$[L+ /10] / [10 *L +/100]$	15
BBIG	L+/10	31007	4,300	$[L+ /10] / [3.3 *L +/33]$	1.4
	L+/33	31008	950	$[L+ /33] / [3 *L +/100]$	3.6
	L+/100	31009	89	$[L+ /10] / [10 *L +/100]$	4.9
PBIGAB	L+/10	41007	6,500	$[L+ /10] / [3.3 *L +/33]$	1.2
	L+/33	41008	1,600	$[L+ /33] / [3 *L +/100]$	4.9
	L+/100	41009	107	$[L+ /10] / [10 *L +/100]$	6.0
BIG	L+/10	61010	940	$[L+ /10] / [3.3 *L +/33]$	2.0
	L+/33	61008	140	$[L+ /33] / [3 *L +/100]$	3.0
	L+/100	61011	16	$[L+ /10] / [10 *L +/100]$	5.9

Figure 2: Neutralizing Potency at ED₅₀ Relative to L+/10 Dilution using PBIGA, PBIGB, BBIG, PBIGAB and BIG for Serotype A



PBIGA and PBIGB appear to have the lowest avidity. However, PBIGAB resulted in higher avidity than either PBIGA or PBIGB. Combining PBIGA with PBIGB allows the highest affinity/avidity antibodies of both IG's to interact, resulting in enhanced overall avidity.

4.0 DISCUSSION

There is substantial evidence that humoral immunity plays a key role in conferring protection against botulinum toxins following vaccination with PBT. Passive immunization with PBT-derived antibodies has been shown to protect numerous animal models from lethal exposures to botulinum toxins (Iakovlev, 1958; Oberst et al., 1967; Lewis and Metzger, 1980; Franz et al., 1993; Gelzleichter et al., 1998a) and PBT-derived antibodies have been successfully used to treat botulism in clinical settings (Frankovich and Arnon, 1991; Arnon et al., 1997). More supporting evidence comes from studies with similar toxin-induced disease where immunological protection has been shown to be humorally mediated (Salyers and Whitt, 1994). MREF Task 97-53 is the last of a series of studies designed to establish and confirm the relationship between the humoral immune response, as measured by NAC in serum, and magnitude of protection from botulinum toxins.

As part of a series of studies (MREF Tasks 97-51, 97-52, and 97-53), the overall program objective was to compare the relative protection conferred by passive transfer of botulinum antibodies derived at different time points subsequent to vaccination. The IG preparation used in Task 97-52 (BIG, BB-IND 1332) was obtained from plasma of volunteers given not only the primary vaccination series but also numerous booster vaccinations. Task 97-53, in contrast, is an identical set of experiments as Task 52, designed to measure protection conferred by IG preparations collected from volunteers approximately four weeks after the primary series (0, 2, 12 weeks) or approximately four weeks after a six month booster vaccination.

There is evidence that qualitative changes in circulating antibodies occur as the immune response matures and that these changes may influence the humoral defense of the host (Narita et al., 1996; Ferreira et al., 1996). Changes in antibody affinity or avidity (Berman et al., 1994; Ahlstedt et al., 1974; Pincus et al., 1988; Hetherington and Lepow, 1992) or relative abundance of IgG subclasses (Ferreira et al., 1996) are known to affect humoral protection. Because of these influences, the use of enzyme-linked immunoassays or other assays that measure specific antibody quantity may not always be an appropriate means for gauging host defenses. The Cardella assay for neutralizing botulinum toxin antibodies in theory can compensate for differences in antibody binding properties; however, values obtained with this procedure are also

influenced by antibody concentration during the testing procedures. For this reason, Tasks 97-51, -52, and -53 were designed to examine the ability of NAC measurements to predict protective efficacy following passive transfer of antibodies obtained at various time points in the vaccination process.

When the protective ratios of the four recent IG preparations (ranging from 7,400 – 13,200) were compared to the protective ratio of BIG (480) determined in Task-52, the protective ratios for all of the new preparations were significantly higher (approximately 15-25 times higher). Reasons for these differences cannot be explained at this time but may be attributed to the following factors:

- Age of IG preparation (BIG was manufactured over twenty years ago);
- Difference in IG manufacturers [SciMedX Corporation (PBIG and BBIG)] vs University of Minnesota (BIG)
- Difference in vaccine lots [PBTA and PBTB (PBIG and BBIG)] vs various lots primarily A-2 (BIG).

The antibody avidity of the IG preparations increased as a function of number of immunizations administered or by pooling of the IG from different PBT lots.

5.0 CONCLUSIONS

The estimated mean serum NAC values attained 24 hours after IG administration were 0.058, 0.057, 0.057, and 0.082 U/mL for PBIGA, PBIGB, PBIGAB, and BBIG, respectively. Clinical profiles for IG (current and historical data) and CIG-pretreated groups were similar for guinea pigs showing signs of botulism. All IG preparations conferred substantial protection with protective ratios of 7,400, 13,200, 9,000, and 11,800, for PBIGA, PBIGB, PBIGAB, and BBIG respectively, against serotype A. There were no significant differences between the protective ratios of PBIGA, PBIGB, PBIGAB, or the pooled BBIG. When the protective ratios of these current IG preparations were compared against historical data (MREF Task 97-52) generated using BIG, the protective ratios for all preparations were significantly higher (approximately 15-25 times higher) than for the BIG. Neutralizing antibody avidity appears to increase as a function of the number of immunizations or by pooling of the IG from different PBT lots. In summary,

NAC correlates well with protection; however, it is not predictive of the magnitude of protection. For example, a 10 U/kg BIG dose was administered to guinea pigs to attain a serum NAC of approximately 0.06 U/mL and demonstrated a protective ratio of 480. However, the same dose of IG preparation prepared from PBT lots A and B was administered to guinea pigs to attain a serum NAC of approximately 0.06 U/mL and demonstrated a protective ratio ranging from 7,400-13,200.

6.0 ARCHIVES

Records pertaining to the conduct of the study are contained in Battelle laboratory record books that are specific to this task. These records and the final report will be archived at Battelle. Samples of human plasma and immune globulin preparations will be returned to the sponsor. Samples of botulinum toxins will be archived at Battelle. After acceptance of the final report, all guinea pig sera samples will be destroyed.

7.0 ACKNOWLEDGEMENTS

The names, titles, and degrees or certification of the principal contributors to this study are listed below:

<u>Name</u>	<u>Title</u>	<u>Degree</u>
Ms. Rebekah Starner	Assistant Study Director	B.S.
Ms. Michelle L. Clagett	Study Supervisor	B.S.
Ms. Nancy A. Niemuth	Statistician	M.A.
Ms. M. Claire Matthews	Statistician	M.A.
Ms. Jennifer R. Holdcraft	Statistician	M.A.S
Dr. Thomas Gelzleichter	Consultant – Genentech, Inc.	Ph.D.

There are a number of people who contributed to the successful completion of this task. Among the many are: Tarin Brown, Amy Forest, Amy Harris, Hulbina Hirst, Jennifer Quick, Emily Syar, and Rob Jarvis for performance on technical tasks and Charlotte Hirst for report preparation.

8.0 REFERENCES

Ahlstedt S, Holmgren J, and Hanson LA (1974). Protective capacity of antibodies against *E. coli* O antigen with special reference to the avidity. *Int. Arch. Allergy Appl. Immunol.* 46, 470-80.

Arnon S, Schechter R, and Hatheway C (1997). Results of a randomized controlled trial of human botulism immune globulin for the treatment of infant botulism. Abstract for the Meeting of the Interagency Botulism Research Coordinating Committee, November 12-14, 1997, Bethesda, MD.

Berman PW, Eastman DJ, Wilkes DM, Nakamura GR, Gregory TJ, Schwartz D, Gorse G, Belshe R, Clements ML, and Byrn RA (1994). Comparison of the immune response to recombinant gp120 in humans and chimpanzees. *AIDS*, 8(5), 591-601.

Cardella MA (1964). Botulinum Toxoids. In KH Lewis and K Cassel, Jr (ed), Botulism: Proceeding of a Symposium. U.S. Department of Health, Education, and Welfare, Public Service, Cincinnati, 113-130.

Ellis RJ (1982). Immunobiologic agents and drugs available from the Centers for Disease Control. Descriptions, recommendations, adverse reactions, and serologic response. 3rd Ed. Centers for Disease Control, Atlanta.

Feder PI, Olson CT, Hobson DW, Matthews MC, and Joiner RL (1991). Statistical analysis of dose-response and iteratively reweighted nonlinear least squares regression techniques. *Drug Information J.* 25, 323-334.

Ferreira MU, Kimura EAS, deSouza JM, and Katzin AM (1996). The isotype composition and avidity of naturally acquired anti-*plasmodium falciparum* antibodies: differential patterns in clinically immune Africans and amazonian patients. *Am. J. Trop. Med. Hyg.*, 55(3), 315-23.

Finney DJ (1971). In Probit Analysis. Third Edition, Cambridge University Press, Cambridge, England.

Frankovich TL and Arnon SS (1991). Clinical trial of botulism immune globulin for infant botulism. *West J. Med.* 154(1), 103.

Franz DR, Pitt LM, Clayton MA, Hanes MA, and Rose KJ (1993). Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism. In Botulinum and Tetanus Neurotoxins. Edited by B.R. DasGupta, Plenum Press, New York, 473-476.

Gelzleichter TR, Myers MA, Menton RG, Niemuth NA, and Matthews MC (1998a). Evaluation of the passive protection against five serotypes of botulinum toxin provided by botulinum human immune globulin in an animal model. Final Report for Battelle Medical Research and Evaluation Facility Task 96-45 submitted to the Department of Defense Joint Program Office for Biological Defense, May 1998.

Gelzleichter TR, Myers MA, Menton RG, Niemuth NA, and Matthews MC (1998b). Correlation of toxicity of botulinum toxins by different routes of administration. Final Report for Battelle Medical Research and Evaluation Facility Task 97-51 submitted to the Department of Defense Joint Program Office for Biological Defense, November 1998.

Hamilton MA, Rusco RC, and Thurston, RV (1977). Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. *Env. Sci. & Technol.* 11(7):714-719.

Hetherington SV and Lepow ML (1992). Correlation between antibody affinity and serum bactericidal activity in infants. *J. Infect. Dis.*, 165, 753-6.

Iakovlev AM (1958). The importance of antitoxin immunity for the defense of the body in respiratory penetration of bacterial toxins. I. The role of passive immunity in the defense of the body against respiratory disease caused by *Clostridium botulinum* toxins. *Zhur. Mikrobiol. Epidemiol. Immunobiol.* 29, 904-9.

Lewis GE and Metzger JF (1980). Studies on the prophylaxis and treatment of botulism. In Natural Toxins D. Eaker and T. Wadstrom, ed., Pergamon Press, Oxford, 601-6.

Narita M, Yamada S, Matsuzono, Itakura O, Togashi T and Kikuta H (1996). Immunoglobulin G avidity testing in serum and cerebrospinal fluid for analysis of measles virus infection. *Clin. Diag. Lab. Immunol.* 3(2), 211-15.

Oberst FW and Crook JW, Cresthull P and House MJ (1967). Evaluation of botulinum antitoxin, supportive therapy, and artificial respiration in monkeys with experimental botulism. *Clin. Pharm. Ther.* 9, 209-14.

Pincus SH, Shigeoka AO, Moe AO, Ewing LP, and Hill HR (1988). Protective efficacy of IgM monoclonal antibodies in experimental group B streptococcal infection is a function of antibody avidity. *J. Immunol.* 140, 2779-2785.

Salyers AA and Whitt DD (1994). Disease without colonization: food-borne toxicoses caused by *Clostridium botulinum*, *Staphylococcus aureus*, and *Clostridium perfringens*. In Bacterial Pathogenesis. A Molecular Approach. Salyers AA, Whitt DD. ASM Press, Washington DC, 130-36.

Siegel LS (1988). Human immune response to botulinum pentavalent (ABCDE) toxoid determined by a neutralization test and by an enzyme-linked immunosorbent assay. *J. Clin. Microbiol.*, 26(11), 2351-6.

APPENDIX A

MREF Protocol 130

**Protection of Guinea Pigs by Passive Immunization with Pooled Human
Immune Globulin Obtained Following Vaccination**

MREF Protocol 130
G155553A
Medical Research and
Evaluation Facility
Effective Date July 17, 1998
Page 1 of 33

**Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin
Obtained Following Vaccination**

STUDY NO. G155553A

Study Performed by Battelle Memorial Institute
Medical Research and Evaluation Facility (MREF)
Bldg. JM-1
State Route 142
Plain City Georgesville Road
West Jefferson, Ohio 43162

MREF MANAGER: James E. Estep, D.V.M., Ph.D.

PRINCIPAL INVESTIGATOR: Carl T. Olson, D.V.M., Ph.D., D.A.B.V.T., D.A.B.T.

STUDY DIRECTOR: Thomas R. Gelzleichter, Ph.D., D.A.B.T

SPONSOR: Joint Program Office for Biological Defense
Fort Detrick, Frederick, MD 21702

SPONSOR MONITOR: LTC Richard R. Stotts, D.V.M., Ph.D., Contracting Officer's
Representative

PROTOCOL DATE: 7/17/98

Approval Signatures


Thomas R. Gelzleichter, Ph.D., D.A.B.T.
Study Director

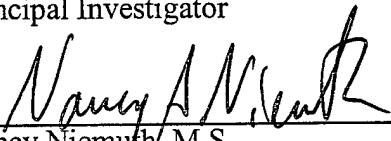
7/15/98
Date


Michelle Clagett, B.S.
Study Supervisor

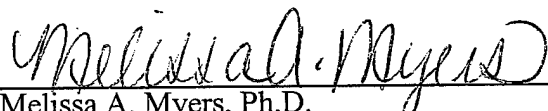
7/21/98
Date


Carl T. Olson, D.V.M., Ph.D., D.A.B.V.T., D.A.B.T.
Principal Investigator

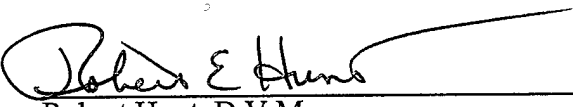
7/20/98
Date


Nancy Niemuth, M.S.
Statistician

7/21/98
Date


Melissa A. Myers, Ph.D.
Microbiologist

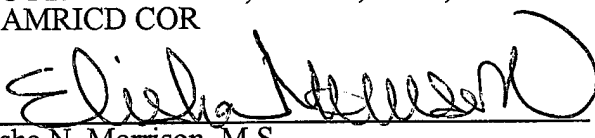
7/21/98
Date


Robert Hunt, D.V.M.
Study Veterinarian

16 July 98
Date


LTC Richard R. Stotts, D.V.M., Ph.D., D.A.B.T.
USAMRICD COR

17 July 98
Date

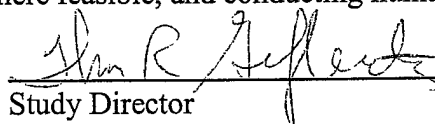

Elisha N. Morrison, M.S.
Quality Assurance Specialist

7/24/98
Date

Assurances: As the Study Director on this protocol, I provide the following assurances:

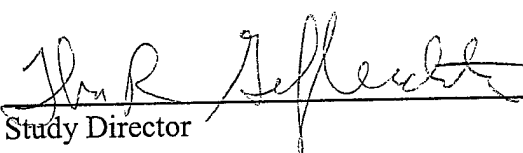
- A. **Animal Use:** The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a deviation is specifically approved by the IACUC.
- B. **Duplication of Effort:** I have made a reasonable, good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.
- C. **Statistical Assurance:** I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."
- D. **Biohazard\Safety:** I have taken into consideration, and I have made the proper coordinations regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, etc., in the preparation of this protocol.
- E. **Training:** I verify that the personnel performing the animal procedures/manipulations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused as a result of the procedures/manipulations.
- F. **Responsibility:** I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" which the DoD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible, and conducting humane and lawful research.

(Signature Required)

 7/15/98
Study Director

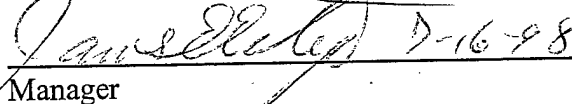
- G. Painful Procedures: I am conducting biomedical experiments that may potentially cause more than momentary or slight pain or distress to animals that will not be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of the proposed experiment.

(Signature Required)

 7/15/98
Study Director

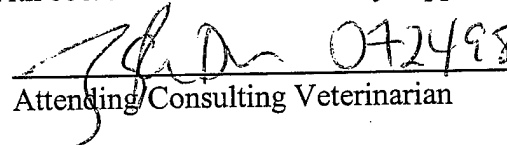
- H. Scientific Review: Signature verifies that this proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

(Signature Required)

 7-16-98
Manager

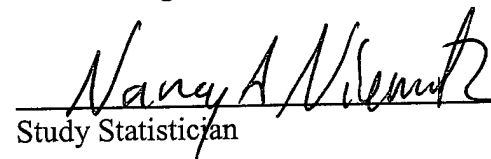
- I. Attending/Consulting Veterinarian: The attending/consulting veterinarian has reviewed the protocol and was consulted in the planning of procedures that require veterinary input, e.g., an unalleviated pain procedure. In addition, the veterinarian/ veterinary medicine department has assisted with coordination for veterinary support to the protocol.

(Signature Required)

 072498
Attending/Consulting Veterinarian

- J. Statistical Review: A person knowledgeable in statistics has reviewed the experimental design.

(Signature Required)

 7/21/98
Study Statistician

This document is a confidential communication of Battelle Memorial Institute. Acceptance of this document constitutes the agreement by the recipient that no unpublished information contained herein will be published or disclosed without the prior written approval of Battelle Memorial Institute, except that this document may be disclosed to appropriate institutional review boards so long as they are requested to maintain its confidentiality.

TABLE OF CONTENTS

	<u>Page</u>
PROTOCOL SUMMARY	8
STUDY FLOW CHART	10
1.0 Introduction	10
2.0 Study Objectives	14
2.1 Primary Objective	14
2.2 Military Relevance	14
3.0 Study Plan	14
3.1 Design	14
3.2 Justification of Design	20
3.3 Population	22
3.3.1 Inclusion Criteria	25
3.3.2 Exclusion Criteria	25
3.4 Subject Assignment to Treatment Group	25
3.5 Dose and Dose Selection	25
3.6 Blinding	25
3.7 Procedures at Each Visit	25
3.8 Premature Withdrawal of Subjects From the Study	26

TABLE OF CONTENTS
(Continued)

	<u>Page</u>
3.9 Study Variables	26
3.9.1 Primary Effectiveness Variable	26
3.9.2 Secondary Effectiveness Variable	26
3.9.3 Safety Variables	26
4.0 Supplies	27
4.1 Test Article	27
4.2 Packaging and Labeling	27
4.3 Storage Requirements	27
4.4 Clinical Supplies Accountability	27
5.0 Trial Methods	28
6.0 Analytical and Statistical Plan	28
6.1 Sample Size	28
6.2 Statistical Methods	28
6.3 Missing Value Handling	30
6.4 Records to be Maintained	30
7.0 References	31

8.0 APPENDICES

- 8.1 Investigators Facilities, and Qualifications
- 8.2 Pharmaceutical Ingredients and Quality
- 8.3 Mouse Bioassays
- 8.4 Summary of USAMRIID Protocol FY92-16

PROTOCOL SUMMARY

TITLE OF STUDY: Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin Obtained Following Vaccination

EXPERIMENTAL PHASE: Protection conferred by treatment with immune globulin isolated from vaccinated volunteers (pentavalent ABCDE botulinum toxoid vaccine) is assessed for parenteral challenge with botulinum toxins. Plasma is obtained from volunteers approximately twenty-eight days past the primary vaccination series and twenty-eight days past a six-month booster vaccination. Antibody avidity is determined for immune globulin preparations.

INVESTIGATORS:

MREF Manager: James E. Estep, D.V.M., Ph.D.

Principal Investigator: Carl T. Olson, D.V.M., Ph.D., D.A.B.V.T., D.A.B.T.

Study Director: Thomas R. Gelzleichter, Ph.D., D.A.B.T.

Study Supervisor: Michelle Clagett, B.S.

Study Statistician: Nancy Niemuth, M.S.

Study Microbiologist: Melissa A. Myers, Ph.D.

Study Veterinarian: Robert Hunt, D.V.M.

PERIOD OF EXPERIMENTS: Approximately from 7/17/98 to 6/30/99

STUDY VARIABLES: Primary study variable is mortality.

DESIGN: The experimental objective is to determine the protection provided by botulinum immune globulin following both the primary vaccination series (PBIG) and a six month booster immunization (BBIG) against the lethal effects of botulinum toxins, as measured by a protective ratio. The guinea pigs are treated with PBIG or BBIG to attain circulating plasma levels of approximately 0.06 U/mL. Control animals receive an identical amount of immune globulin from a control population (CIG), normalized to total protein content. Twenty-four hours after these treatments, animals are challenged with the analogous serotype of botulinum toxin by a parenteral route. LD₅₀ values are determined by a stage-wise approach (up to four stages total) for PBIG-, BBIG- and CIG-treated animals. In the first stage, small numbers of animals are

treated with variable amounts of toxin to obtain a rough approximation of the LD₅₀. Survival information is used to design each subsequent stage of experiments. The protective ratio is determined as the LD₅₀-treated: LD₅₀-untreated.

INCLUSION CRITERIA: Not applicable to study.

EXCLUSION CRITERIA: Animals suffering from nonstudy-related illnesses will be removed from the study at the discretion of the Study Director and/or the Study Veterinarian.

NUMBER OF ANIMALS:

1. male guinea pigs: 832
2. male mice: 27,809

BIOLOGICAL AGENTS/TEST ARTICLES:

1. Biological Agent: Partially purified botulinum toxins A, B, C, D, and E are produced in the laboratories of Dr. Eric Johnson at the University of Wisconsin. Appropriate identification (batch and lot numbers) is provided with each shipment. Toxin potency has been verified by mouse intraperitoneal LD₅₀ (MIPLD₅₀) assay. Serospecificity has been verified by mouse neutralization assay. Protein content has been determined by a colorimetric assay. Protein heterogeneity has been assessed by gel electrophoresis.
2. Test/Control Articles: Botulism Immune Globulin (PBIG and BBIG) is purified from human plasma by Cohn (method 6) - Oncley (method 9) cold ethanol fractionation. Plasma is obtained from volunteers enrolled in clinical trials with Pentavalent (ABCDE) Botulinum Toxoid, Aluminum Phosphate Adsorbed (BB-IND 3723, Lot 003 and 004) at approximately twenty-eight days following the primary vaccination series (day 112 time point) and at 28 days following a six month booster vaccination. To serve as a control article, human immune globulin is obtained from volunteers prior to vaccination. Before use on study, IgG, IgM, and IgA concentrations will be determined by radial immunodiffusion (RID); relative abundance of IgG subclasses (1-4) will be determined by enzyme-linked immunosorbent assay (ELISA); and protein content will be determined by colorimetric assay.

DURATION OF TREATMENT: Animals are observed for fourteen days following toxin exposures.

DRUG FORM, ROUTE OF ADMINISTRATION AND DOSE REGIMEN: PBIG and BBIG are sterile solutions of human immune globulin purified from plasma by Cohn (method 6) Oncley (method 9) fractionation. These products are derived from pooled adult human plasma from volunteers immunized with pentavalent (ABCDE) botulinum toxoid. PBIG and BBIG will be administered by intraperitoneal injection at a dosage of 10 U/kg. CIG is processed from human plasma from a control (non-vaccinated) population in an analogous fashion as was used for PBIG preparations. CIG treatments will be by intraperitoneal injection. Dosages are normalized to equal the protein content of PBIG dosages.

STUDY FLOWCHART (APPROXIMATE DATES)

Phase I

1. Stage 1: Validation of assays/standards (Prior to use on study)	June 1998
2. Stage 2: PBIG avidity experiments	November 1998
3. Stage 3: PBIG efficacy experiments	November 1998
4. Stage 4: Preliminary report for Phase I	April 1999

Phase II

5. Stage 5: BBIG avidity experiments	March 1999
6. Stage 6: BBIG efficacy experiments	March 1999
7. Stage 7: Final report completed	July 1999
8. End of study	July 1999

1.0 INTRODUCTION

Non-technical Synopsis: Experiments are currently underway at Battelle MREF to establish the mouse neutralization assay as a serological correlate for protection against exposure to botulinum toxins. These studies are in support of licensure of a pentavalent (ABCDE) botulinum toxoid vaccine (BB-IND 3723). A previous study (MREF protocol G155545A) was designed to assess protection against an aerosolized threat of botulinum toxins in animals passively immunized with human botulinum antibodies. The botulinum immune

globulin product (BIG) used in this study was obtained from volunteers vaccinated with the pentavalent botulinum toxoid vaccine. Originally intended as a therapeutic agent (BB-IND 1332), BIG was obtained from plasma of volunteers that had received the primary vaccination series and numerous booster vaccinations. Concerns have been raised that the avidity (binding strength) of botulinum antibodies obtained in this manner may differ significantly from that of botulinum antibodies obtained very early in the vaccination process. The military, however, may have to vaccinate troops shortly before deployment into high risk areas. With this in mind, these tests are designed to establish efficacy at two time points, shortly after the primary series and shortly after a 6-month booster vaccination. As a new clinical trial for the pentavalent botulinum toxoid vaccine is scheduled to begin later this year, it is now possible to obtain botulinum antibodies from vaccinated volunteers at early time points in the vaccination process. The objectives of this study are to determine the level of protection afforded by passive immunization of an animal model with human antibodies obtained approximately 28 days following the primary vaccination series or following the 6 month booster and to compare differences in avidity between the BIG, PBIG, and BBIG preparations. The experiments are designed in a manner that allows direct comparison with protection afforded by BIG preparations as demonstrated in MREF protocol G15552A.

- A. Background: Pentavalent (ABCDE) botulinum toxoid vaccine is intended for use as a prophylactic agent for countering threats from botulinum toxins. Preclinical studies have demonstrated a high degree of efficacy in animal models but very little data are available for human efficacy. The overall objective of this research is to establish a correlation between neutralizing antibodies and protection against botulinum toxins. The mouse neutralization assay was first developed by Cardella and coworkers at the Biological Laboratories at Fort Detrick in the early 1960s (Cardella, 1964). Since this time, it has served as the principal assay for measuring neutralizing antibody levels in serum. The U.S. Army proposes to use this assay as part of clinical trials to serve as a serological correlate for efficacy in humans. In the following study, guinea pigs are passively immunized with PBIG and challenged with botulinum toxins administered by a parenteral route (route was determined in study G15551A). Level of protection is expressed as a ratio of LD_{50} -protected: LD_{50} -unprotected.

Related Studies: Experiments conducted at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) have demonstrated that primates immunized with botulinum toxoid vaccine (pentavalent ABCDE) were completely protected against aerosol exposures to botulinum A at dosages equivalent to approximately 250 times the LD_{50} for untreated primates (unpublished). Although few animals were exposed at this level, the protective ratio (defined as the LD_{50} -treated: LD_{50} -untreated) was estimated to be 7043. To a lesser extent, monkeys passively immunized with either human or

equine antitoxin immune globulin were also protected from aerosol exposure to botulinum A. Seven monkeys, passively immunized with human BIG and having serum antibody levels ranging from 0.11 - 0.22 IU/mL, survived lethal aerosol exposures (target exposure of 6 x LD₅₀). An additional monkey, passively treated six weeks prior, had no detectable antibody level (<0.02 IU/mL) but still survived a lethal challenge (Franz et al., 1993). In postexposure treatment studies using equine immune globulin, four out of four monkeys exposed to a range of aerosol concentrations (6 - 43 x monkey LD₅₀) were protected when treated with 14 IU/kg equine immune globulin 24 hr after exposure. In addition, three out of four exposed to similar concentrations were protected when treated with 143 IU/kg (unpublished USAMRIID protocol # FY9216, see Appendix 8.4). Based on these latter results, 24 hr postexposure treatments with equine immune globulin were estimated to yield a protective ratio of 124 (McNally et al., 1994). Botulinum immune globulin preparations, raised in both guinea pigs and horses, were able to protect guinea pigs from lethal botulinum challenges by an IP route administered twenty four hours prior to toxin challenge. However, only homologous (guinea pig) product could protect when administered eighteen days prior to toxin challenge (Lewis and Metzger, 1980). Although a substantial database has been gathered on the protective effects of pentavalent toxoid vaccine and/or immune globulin to botulinum A aerosols, little data are available for other botulinum serotypes.

B. Literature Search:

1. MEDLINE 1966-1995 (10/31/95); 1996-1997 (6/25/97)
2. FEDRIP (11/1/95); 1996-1997 (6/25/97)
3. DTIC 1963-1993 (11/1/95); 1996-1997 (6/25/97)
4. AGRICOLA 1970-1995 (11/2/95); 1996-1997 (6/25/97)
5. Current Contents 1990-1995 (11/2/95); 1996-1997 (6/25/97)
6. BIOSIS 1969-1995 (11/2/95); 1996-1997 (6/25/97)

Keywords:

1. Searches for duplication of previous research (MEDLINE, FEDRIP, DTIC, AGRICOLA, Current Contents, BIOSIS): Botulism, botulinum toxins, immune globulin, IgG, vaccine, inhalation, aerosol.
2. Searches for alternatives to painful procedures (MEDLINE, AGRICOLA, Current Contents, BIOSIS): Botulism, botulinum toxins, analgesics, anesthetics, pain, alternative procedures, drug therapy, analytical methods.

Synopsis of Findings:

An initial literature review was conducted in 1995 for two related studies (MREF protocols G155539A and G155545A). In June 1997, these reviews were updated for the period 1995 to 1997 (June).

None of the literature searches identified nonanimal methods for testing the efficacy of botulinum immune globulin in preventing botulinum-induced death. By the nature of the scientific objective, an *in vivo* system is necessary to test the ability of immune globulin to prevent death. A search of therapeutic treatments for human victims of botulism did not identify any methods of pain alleviation such as analgesia, anesthesia or sedation. Such methods are contraindicated when administered with agents known to cause respiratory paralysis.

A search for alternatives to the mouse assays for botulinum toxins and antitoxin antibodies revealed that numerous ELISA methods have been developed (Ekong, et al., 1995). ELISAs are commonly used to detect botulinum toxins in biologic samples and food items. However, in a study performed at USAMRIID an ELISA assay was compared head-to-head with the established neutralization assay for detection of antibodies to botulinum A and B and did not show a high degree of correlation (correlation coefficients of 0.69 and 0.77, respectively). In the words of the authors "due to the wide dispersion of values obtained, using ELISA results to predict neutralizing antibody levels is unwarranted" (Seigel, 1988). It is thought that the lack of correlation stems from the inability of ELISA methods to distinguish between neutralizing and non-neutralizing antibodies, with the former type being the most important for protection. The mouse assays directly measure toxin potency whereas the ELISA only measures specific toxin epitopes. As the potency of botulinum toxins can vary greatly between batches, it is critical that toxin contents are based on potency rather than mass concentration, which is the critical parameter. Dr. George Doellgast, at Wake Forest University Medical Center, is currently developing an enzyme-linked coagulation assay (ELISA-ELCA) which has pushed the level of sensitivity down to a level found in the mouse assays (5-10 pg/mL). However, this assay (1) still measures concentration and not potency, (2) has not been developed for all five botulinum toxins used in this protocol, and (3) is not yet commercially available (personal communication with the author, Doellgast et al., 1993, 1994). Due to the inherent limitations of immunoassays, the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) still consider the mouse bioassays to be the standard assays for reporting antibody titers and toxin potencies. An alternative mouse bioassay also exists for measuring botulinum therapeutic potency (Pearce et al., 1995); however, therapeutic potential does not necessarily correlate with lethality (LD_{50}) and is considered a less relevant assay given the goals of the proposed work.

2.0 STUDY OBJECTIVES

2.1 Primary Objective

The objective of the proposed research is to determine the level of protection afforded by treatments with PBIG and BBIG in protecting guinea pigs against challenges with botulinum toxins A, B, C, D, and E. This study will be conducted following the guidelines of the Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations (21 CFR 58).

2.2 Military Relevance

Botulinum toxins are recognized as biological warfare threats that can cause illness and death in unprotected soldiers. Currently, no FDA-licensed vaccines are available for field use. This work supports the efficacy assessment of pentavalent botulinum toxoid that is currently an investigational new drug.

3.0 STUDY PLAN

3.1 Design

A. Pooling of Plasma: Plasma samples from vaccinated volunteers (JPO-BD sponsored study entitled "Phase I/II evaluation of safety and immunogenicity of pentavalent botulinum toxoid (A-E) administered to healthy volunteers") are blended prior to purification. For PBIG plasma, samples will be screened by mouse neutralization assay before blending to eliminate nonresponders (i.e., those volunteers without detectable botulinum antibodies). As it is anticipated that BBIG plasma will have higher average antibody levels, nearly all of which should contain measurable botulinum antibodies, no prescreening will be done to remove nonresponders. Plasma is segregated into five separate pools based on the lot of vaccine received.

1. Study day zero prebleed (prior to vaccination)
2. 4 weeks after primary series (vaccine lot -003)
3. 4 weeks after primary series (vaccine lot -004)
4. 4 weeks after 6 month boost (vaccine lot -003)
5. 4 weeks after 6 month boost (vaccine lot -004)

B. Immune Globulin Purification: Prior to use on study, immune globulin is prepared from plasma by Cohn (method 6) - Oncley (method 9) fractionation. Samples are assayed by the mouse neutralization assay before and after purification procedures

to determine relative recovery of (1) neutralizing antibodies for serotypes A-E, (2) protein, (3) IgG, (4) IgM, and (5) IgA, and (6) IgG subtypes 1-4. For the above assays, samples are analyzed in duplicate and the values averaged. Neutralizing antibody, Ig, and protein determinations are conducted as per Battelle SOP requirements.

- C. Avidity Determinations: To characterize the avidity of BIG, PBIG, and BBIG preparations the following procedures are used. Avidity determinations will be conducted at L+/10, L+/33, and L+/100 doses for vaccine lots 003 and 004 (both PBIG and BBIG) and also for BIG. Avidity is determined for serotypes A-E. L+/x is defined as a challenge dosage of toxin that when mixed with 1/x units of antibody and injected into mice can kill 50 percent of test animals within 96 hours. Fifty percent lethality is estimated by probit analysis using graded dilutions of toxin mixed with fixed amounts of immune globulin. The final L+/x and immune globulin mixture is delivered to mice (17-23 g weight range) in a 0.5 mL volume. Toxin and antibody preparations are diluted in 0.2 percent gelatin phosphate buffer (GPBS, 0.2 percent gelatin, 0.4 percent dibasic sodium phosphate, pH 6.2). For each L+/x dosage, a broad-based range finding curve is constructed using two mice per group (ten groups total) to determine a rough approximation of the LD₅₀. Once an approximate range is established, the avidity is determined with a single curve using the following criteria:

1. Weight range on day of experiment is 17-23 g
2. Minimum number of mice per group: 5
3. Dose ranges will sequentially increase by approximately thirty percent between intervals

- D. Determination of LD₅₀ for three botulinum serotypes in animals pretreated with PBIG. Test species: Guinea pig.

To characterize the level of protection conferred by PBIG (or BBIG) treatment, the protective ratio (defined as LD₅₀-protected: LD₅₀-unprotected) will be determined for three botulinum toxin serotypes. It is anticipated that the yield of plasma antibodies will not be sufficient to conduct these studies for all five serotypes of interest (A-E). Experiments are restricted to serotypes A, B, and E, and to the plasma pool yielding the highest neutralizing antibody levels (i.e., lot 003 or 004). The LD₅₀-unprotected is defined as the estimated dosage of toxin at which 50 percent of CIG-treated animals die within fourteen days. Likewise, the LD₅₀-protected is defined as the estimated dosage of toxin at which 50 percent of PBIG(BBIG)-treated animals die within fourteen days.

Immune Globulin Pretreatment: Guinea pigs are pretreated with PBIG, BBIG, or CIG twenty-four hours prior to toxin treatment. PBIG and BBIG are administered at a dosage of 10 U/ kg. CIG dosages are normalized to the protein content of the BIG or BBIG dosage. Based on the results of Task 96-45 (protocol G155545A), circulating antibody levels at 24 hours postadministration is expected to be at an approximate level of 0.06 U/mL, a level that is above the limit of quantitation for all five serotypes.

NOTE: 0.06 U/mL corresponds to the approximate target levels for phase 3 iteration 2 experiments for protocol G155545A ($0.25 \text{ U/mL} \div 4 = 0.06 \text{ U/mL}$). A somewhat lower initial target has been selected due to the lower yields of antibody anticipated in this study.

Route of Toxin Administration: Botulinum toxins are administered by an IM route. Botulinum toxins are administered to guinea pigs at 24 ± 1 hours after the administration of immune globulin. Toxin samples are diluted with a gelatin phosphate-buffered solution (GPBS, 0.2 percent gelatin, 0.4 percent dibasic sodium phosphate, pH 6.2).

LD₅₀ values are determined using a stage-wise approach. Small numbers of animals are treated in each of several sequential iterations. As iterations are completed, the Study Director, in consultation with the Study Statistician, will select dosages for the next iteration, making use of all available information from previous iterations. For each serotype, experiments will be conducted in up to four iterations.

For the first iterations, dosages are selected based on the results of Task 97-51 (Protocol G155551A) and Task 97-52 (Protocol G155552A). For CIG-treated control groups, six guinea pigs are treated with six different dosages. These dosages are selected to fall in the range around the estimated LD₅₀ for toxin-exposed guinea pigs as determined in Task 97-51 for the selected route of administration. For PBIG or BBIG-treated groups, six guinea pigs are treated at dosages that fall around the estimated LD₅₀, as determined in Task 97-52. Test levels are chosen by the Study Director, in consultation with the Study Statistician, and based on such factors as the probit slope, LD₅₀ and confidence interval for the LCt₅₀ estimation. Up to 24 guinea pigs are tested in iterations two and three for CIG- PBIG- and BBIG- treated groups for each serotype. Dosages are selected based on all available information. If after the first three iterations the half width of the confidence interval is larger than 50 percent then up to 10 additional animals will be dosed in

the fourth and final iteration.

In all cases, guinea pigs are observed for fourteen days following toxin treatment, at the end of which they are euthanatized. During this period, clinical signs and mortality are tabulated. Serum samples are obtained from all animals that survive the fourteen-day period and are stored frozen at approximately -20 degrees C or lower. Samples are destroyed following acceptance of final report by the client.

At each iteration and for each serotype, additional groups of four guinea pigs are treated with each immune globulin preparation to determine average circulating serum antibody content. At the time of toxin treatment (± 1 hr), serum samples are obtained for analysis of neutralizing antibody content. Serum samples are assayed using the neutralization assay according to Battelle SOPs (see Appendix 8.3). To characterize toxin potency, a mouse potency assay (see Appendix 8.3) is conducted at each iteration and dosages are expressed in MIPLD₅₀ units.

Botulinum toxin serotype E is trypsin-treated prior to administration. On the day of administration, toxin samples are first treated using the following procedure: Total protein is adjusted to 0.5 mg/mL either by dilution with PBS or by addition of bovine serum albumin. After trypsin is added at a concentration of 100 μ g/mL, the sample is incubated at 37 ± 3 degrees C for one hour. Trypsin activity is stopped by addition of soybean trypsin inhibitor at a final concentration of 1 mg/mL.

E. Technical Methods:

1. Pain: In previous botulinum studies, the primary clinical signs are lack of response to external stimuli, flaccid paralysis, dyspnea and death, the line separating dyspnea from death being difficult to discern. As it is difficult to judge how much pain or distress is involved with botulinum toxicity it is assumed that pain and/or stress are present.

Pain Categories (animal number, percent):

Mice

- i. No Pain (USDA Code N): 13905 (50 percent)
- ii. Alleviated Pain (USDA Code D): 0 (0 percent)
- iii. Unalleviated Pain or Distress: 13904 (50 percent)

Guinea pigs

- i. No Pain (USDA Code N): 416 (50 percent)
 - ii. Alleviated Pain (USDA Code D): 0 (0 percent)
 - iii. Unalleviated Pain or Distress: 416 (50 percent)
2. Anesthesia, Analgesia, and Tranquilization: Anesthetics, analgesics or tranquilizers cannot be used for the relief of pain, or anxiety in the post-treatment phase due to potential interference with the biological effects of the challenge agent or therapy product. External stimuli and manipulation are minimized to decrease any associated anxiety.
3. Painful Procedure/Paralytics Justification: Botulinum toxins, by the nature of their mechanism of action, are considered paralytic agents. As the cause of death in botulism is respiratory failure, anesthetics cannot be used to alleviate distress as they also act as respiratory depressants that could potentiate the toxic action of the biological agent. Opioid analgesics and barbiturate sedative-hypnotics both can cause respiratory depression. Benzodiazepines, while having fewer effects on respiration, have been shown to have substantial respiratory interactions when used in combination with neuroleptic agents. Because of these side effects, anesthetics, analgesics and sedatives could potentiate the toxicologic effects of botulinum toxins.
4. Painful Procedure Justification: No *in vitro* models of botulinum-induced toxicity that adequately duplicate the *in vivo* effects of botulism are available. The efficacy studies must be performed in animals. At present, the complex nature of the immune response and its protective role against botulism cannot be investigated in any other way. The Study Director has consulted with the Study Veterinarian in the planning of procedures involving unalleviated pain. All rodents challenged with botulinum toxins will be euthanatized at the end of the observation period.
5. Prolonged Restraint: No prolonged restraint is used in this protocol.
6. Surgery: No surgery is performed in this protocol.
7. Quality Control: Shipments will be screened by routine serology for various diseases according to Battelle SOPs. Guinea pigs are screened for pneumonia virus and Sendai virus. Mice are screened for pneumonia virus, reovirus three, Theiler's encephalomyelitis, minute virus, polyoma, ectromelia, Sendai, mouse hepatitis virus, lymphocytic choriomeningitis, mouse adenovirus, K virus and enzootic diarrhea.

E. Animal Manipulations:

1. Blood Sampling: Blood specimens from guinea pigs are collected from the vena cava and/or by cardiac puncture with a syringe following terminal anesthesia with pentobarbital or other suitable anesthetics. Samples are collected and transferred into red-topped Vacutainers® (containing no anticoagulant) or other appropriate containers. Following clotting, serum is separated out, divided into aliquots, and frozen at approximately -20 degrees C or lower for subsequent analysis.
2. Administration Procedures: Guinea pigs are administered immune globulin by IP injection using syringes capped with 3/4"- 20 gauge needles or other appropriately sized needles.
3. Toxin Potency and Antibody Determination: Toxin potency is determined in mice by measuring lethality following intraperitoneal injection. Levels of neutralizing antibodies are determined by mixing graded dilutions of serum or plasma with fixed amounts of toxins. Mice are treated with these mixtures by intraperitoneal injections and monitored for lethality. Procedures are as described in Battelle SOPs (see Appendix 8.3).
4. Animal Identification: Guinea pigs are tagged or marked on the ear to retain positive identification. As the endpoints for mouse assays are percent lethality per treatment group, mice are identified by group only. Once mice are treated and assigned to home cages, living mice will not be moved to different caging during the 96 hour observation period. Procedures to avoid mixing of mice will be carried out as per Battelle SOPs.
5. Behavioral Studies: None
6. Adjuvants: None
7. Clinical Observations: Treated guinea pigs are observed twice daily for the following signs:
 - (a) Ruffled fur
 - (b) Labored breathing
 - (c) Droopy eyelids

- (d) Weakness in limbs
- (e) Total paralysis

F. Veterinary Care:

1. Husbandry Considerations:

- (a) Attending Veterinary Care: Animals will be observed by laboratory animal facility personnel at the beginning and end of each working day for morbidity and moribundity. Discomfort and injury will be limited to that which is unavoidable in the conductance of scientifically valuable research.
- (b) Housing: During and following quarantine, guinea pigs and mice are housed in polycarbonate or stainless steel cages in stainless steel racks equipped with automatic watering systems.
- (c) Lighting: The light/dark cycle will be approximately 12 hours each per day, using fluorescent lighting.
- (d) Temperature: Animal room temperatures will be maintained according to Battelle SOP requirements. Temperatures will be recorded twice daily.
- (e) Humidity: The relative humidity of animal rooms is maintained according to Battelle SOP requirements and recorded twice daily.
- (f) Diet: For guinea pigs, PMI Certified Guinea Pig Chow ® pellets are available *ad libitum*. For mice, PMI Certified Rodent Chow ® is available *ad libitum*. No contaminants that would interfere with the results of the study are known to be present in the feed. Certified analyses of feed are kept as part of the Study Record.
- (g) Water: Water is supplied from the Battelle water system and given *ad libitum* during quarantine and holding. Water is analyzed at a minimum once per year. Analysis includes, but is not limited to, bacterial content, coliform and heterotrophic bacteria counts, water hardness, pH, ion concentration, and heavy metal content. No contaminants that would affect the results of the study are known to be present in the water.

3.2 Justification of Design

- A. Use of the Neutralization Assay: Pentavalent (ABCDE) botulinum toxoid is intended for use as a vaccine for protection of combat troops exposed to botulinum toxins. Efficacy, normally assessed during Phase 3 clinical trials, must be established before this product can be licensed by the FDA. Although clinical trials

can be used to induce antigenic responses in test subjects, Phase 3 clinical trials cannot be used to assess efficacy for this product. Natural incidences of human botulism are extremely rare, rendering field trials of little or no use in assessing efficacy for a vaccine and, given the extreme danger of botulinum toxins, clinical exposures to botulism cannot be conducted. The Centers for Disease Control and Prevention (CDC) reports that only approximately twenty cases of food borne botulism and approximately seventy cases of infant botulism are recorded in the United States annually (CDC, 1994). For these reasons, studies are designed to establish neutralizing antibodies in serum as a serological correlate for efficacy in humans.

- B. Animal Selection: Botulinum toxins have been shown to be potent paralytic agents in a wide array of vertebrates as well as some invertebrate species, although the relative sensitivity can vary greatly between species. Mice and guinea pigs have been shown to be highly sensitive to all five types of botulinum toxin employed in this study (Gill, 1982; Lewis and Metzger, 1980; United States Army Research Institute of Infectious Diseases (USAMRIID), unpublished) and the guinea pig has been shown to be a sensitive model for botulinum toxins delivered via an aerosol route (Cardella, 1964; USAMRIID, unpublished). Although no aerosol exposures are included in this protocol, data will be directly compared with previously conducted inhalation studies (MREF protocol G155545A).
- C. Nonanimal Alternatives Considered: The lethality induced by botulinum toxins is thought to stem from inhibition of neuronal transmission of acetylcholine between synapses rather than from direct toxicity on cells. This leads to a series of events that culminates in multiple organ failure and death. There are no *in vitro* systems available that mimic the complex interactions involved. In addition, there are currently no nonanimal alternatives available that satisfactorily measure botulinum toxin potency or botulinum neutralizing antibodies. Methods using enzyme-linked immunosorbent assays (ELISAs) are being developed but are at present considered of limited use as they can neither measure potency nor discern between neutralizing and non neutralizing antibodies (Seigel, 1988; Ekong et al., 1995) and are not as sensitive as the mouse neutralization assay (Shone et al., 1986).
- D. Refinement, Reduction, Replacement:
1. Refinement: None
 2. Reduction: An iterative approach to guinea pig LD₅₀ determination is employed to insure that dosages are selected in a range that will yield the greatest statistical information while using a smaller number of animals.

3. Replacement: None

3.3 Population

Animals: Male Cr1: (HA) BR guinea pigs (Cavia porcellus) are used for establishing LD₅₀ values. Male Cr1: CD-1 (ICR) BR white mice (Mus musculus) are used for bioassays. Rodents are supplied by Charles River Laboratories (USDA Vendor # 14B013) or other suitable USDA-approved vendors.

TABLE 1. ESTIMATED ANIMAL USAGE

Serotype DR Experiments							
Experiment	Test Guinea Pigs		Control Guinea Pigs		MIPLD ₅₀ (mice) ‡	Neut. Assay (mice)	Avidity Experiments (mice)
	Toxin Exposed	Antibody Exp.	Toxin Exposed	Antibody Exp.			
First serotype (PBIG)	30 - 40	12 - 16	30 - 40	12 - 16	120 - 160	696	
Second serotype (PBIG)	30 - 40	12 - 16	30 - 40	12 - 16	120 - 160	696	
Third serotype (PBIG)	30 - 40	12 - 16	30 - 40	12 - 16	120 - 160	696	
First serotype (BBIG)	30 - 40	12 - 16	30 - 40	12 - 16	120 - 160	696	
Second serotype (BBIG)	30 - 40	12 - 16	30 - 40	12 - 16	120 - 160	696	
Third serotype (BBIG)	30 - 40	12 - 16	30 - 40	12 - 16	120 - 160	696	
QC *			16		24	50	
Repeat Experiments †			144		240	2400	
Lot 003,004 Characterization ††						5800	
Avidity Characterization †††							7254
Characterization of Antibody Stds ††††						3456	889
SemiAnnual Assay Evaluation ‡‡						2560	
Total			664 - 832		984 - 1224	16906	8143

- ‡ Up to forty mice are required for each mouse potency assay. This includes thirty mice for probit analysis and five mice each for negative and positive control groups. For neutralization assays, up to forty-two mice are used for probit analysis of unknown samples. Up to sixty-four mice are used for standard curves (includes three groups of eight for positive and negative control animals).
- * Rodent shipments are screened by routine serology for purposes of quality control.
- † Extra animals are allocated for cases where animals need to be excluded from the study or assays need to be repeated.
- †† For each lot, antibody content is determined before and after plasma processing using a single broad range finding curve followed by two narrow range curves (ran in duplicate on different days and averaged) for all five serotypes. Broad range curves will use ten groups of two plus three control groups (buffer, toxin, antitoxin) of three for a total of 29 mice. For narrow range assays, up to forty-two mice are used per curve. In addition, ten standard curves of up to 64 mice per curve are required (includes three groups of eight for positive and negative control animals).
- ††† Avidity determinations will require seventy-five range finding (broad range) and seventy-five narrow range lethality curves (5 serotypes x five lots of immune globulin x three L+/x levels = 75). Each broad range curve consists of ten doses at two mice per dose plus toxin, antitoxin and buffer control groups (3 each) for up to a total of 29 mice per curve. Narrow range curves consist of seven groups at 5 mice per group plus three control groups for fifty mice per experiment. Serotype E requires an additional trypsin control group ($15 \times 3 + 15 \times 5 = 120$ mice). An extra 20 percent of total required are included in this number for any repeat experiments that may be necessary.
- †††† PerImmune, Inc. 1998 standards will be phased into use during this protocol. As previous studies have used PerImmune 1996 standards, these must be checked for differences in potency, serospecificity and avidity. Standard curves (64 mice) will be run in quadruplicate for both sets of standards to determine relative potency between the two sets of standards. Standard avidity determinations will also be performed on the new standards. As per federal regulations (21CFR58) these standards will be checked every six months for stability ($3 \text{ serotypes} \times 4 \text{ replicates} \times 64 \text{ mice} \times 2 \text{ six-month tests} = 1536 \text{ total}$).
- ‡‡ As per SOP X-014 requirements, the toxin challenge dose must be reevaluated periodically to maintain assay integrity. It is anticipated that two evaluations will be required during the course of Task 97-53. This will entail testing in quadruplicate (up to 64 mice) for all five serotypes. As the challenge doses for some of the serotypes are due to be reoptimized in late July, at approximately the same time the new standards will be evaluated, this requirement will be extended for two months to allow the new standards to be evaluated before the reoptimization takes place.

3.3.1 Inclusion Criteria

Only mice between 18 - 22 and 17 - 23 grams the day of treatment are placed on study for mice lethality and neutralization assays, respectively (weights rounded to three significant figures). Guinea pigs will weigh approximately 375 to 475 grams the day of treatment. Ages are approximately 18 and 35 days old for guinea pigs and mice, respectively.

3.3.2 Exclusion Criteria

Mice and guinea pigs are observed by a staff veterinarian following a minimum of three days quarantine. Only animals free of malformations and clinical signs of disease are used. Only rodents meeting the specified weight restrictions are placed on study. At the discretion of the Study Director or a Battelle veterinarian, animals judged to be abnormal will not be placed on study. Reasons for removal from study will be documented and will become part of the study record.

3.4 Subject Assignment to Treatment Group

Randomization of Animals: Guinea pigs are randomly distributed to experimental groups using a computer-generated randomization algorithm. Mice will not be randomized for lethality assays but only those mice falling within a narrow weight range will be placed on study. In cases where more animals meet acceptance criteria than are needed for a given experiment, animals will be selected in an arbitrary fashion from the available pool until the required number have been selected.

3.5 Dose and Dose Selection

First iteration experiments are designed such that selected dosages are arranged around the anticipated LD₅₀. These numbers are based on previously attained estimates for botulinum LD₅₀ values and LD₅₀ values in guinea pigs pretreated with BIG (MREF protocols G155551A and G155552A).

3.6 Blinding

Not applicable to this study

3.7 Procedures at Each Visit

Not applicable to this study

3.8 Premature Withdrawal of Subjects from the Study

Animals that develop nonstudy related illness will be evaluated by a Battelle Veterinarian for determination of treatment disposition. Under such conditions and in the opinion of the Study Director or a Battelle Veterinarian an animal that is in a moribund state will be euthanatized. No treatment will be given for study-related illness.

3.9 Study Variables

3.9.1 Primary Effectiveness Variable

Study Endpoint: As botulism-induced lethality is the primary concern in humans, the primary effectiveness variable is mortality. Since the primary sign of botulism is flaccid paralysis, discerning between mildly affected and moribund animals is difficult. Guinea pigs are euthanatized by barbiturate overdose followed by exsanguination. Mice are euthanatized by CO₂ overdose or by barbiturate overdose followed by exsanguination. Guinea pigs and mice are euthanatized immediately following observation periods of fourteen and four days, respectively. In cases where the end of the observation period falls over a weekend or a holiday, animals may not be euthanatized until the next working day if they are not demonstrating clinical signs of botulinum toxicity.

3.9.2 Secondary Effectiveness Variable

Not applicable to this study.

3.9.3 Safety Variables

Clinical Safety Variables: Not applicable to this study.

Other Safety Variables:

Containment Level - Following toxin treatment, the site of injection is decontaminated with a hypochlorite solution (approximately 3000 ppm free chlorine or another accepted decontaminant solution). All animals that die or are euthanatized are double bagged and autoclaved before removal from the laboratory facility.

Biohazard Safety - Personnel handling exposed guinea pigs will wear

appropriate garb as described in Battelle SOPs.

Agents Used in this Protocol - Botulinum toxins A, B, C, D, and E (see Background section). Workers with a potential for exposure are vaccinated with pentavalent botulinum toxoid (A-E) vaccine. Toxins will be manipulated in a class II or class III biosafety cabinet and injected into animals following Battelle SOPs. All procedures are performed following the Battelle MREF Biofacility Safety Plan (FSP) document and Battelle SOPs.

Other toxic chemicals to be used include paraformaldehyde, sodium hypochlorite, hydrogen peroxide, soybean trypsin inhibitor, hydrochloric acid, sodium hydroxide, and phosphoric acid.

4.0 SUPPLIES

4.1 Test and Control Articles

Human plasma (PBIG and BBIG) is obtained from volunteers enrolled in clinical trials with the pentavalent (ABCDE) toxoid vaccine (BB-IND 3723, Lot 003 and 004). Prevacination plasma is used to produce control article (PBIG).

4.2 Packaging and Labeling

Not applicable to this study.

4.3 Storage Requirements

See Appendix 8.2. Materials that are designated to be frozen or refrigerated will be stored according to Battelle SOPs. Upon reaching an expiration date, toxin preparations will be reassessed for viability and will be given a new expiration date if deemed to be acceptable for the study.

4.4 Clinical Supplies Accountability

- A. All botulinum toxins are stored in a limited access locked freezer (-70 degrees C. or lower as per Battelle SOP requirements for frozen samples). Toxin samples are periodically analyzed to determine preparation stability.
- B. The Study Director or a designee will maintain accurate records for the receipt of all study toxins, including dates of receipt, and dispensing of materials.

- C. All test agents and standards will be stored in limited access areas. The Study Staff will keep cumulative inventory and dispensing records.

5.0 TRIAL METHODS

A description of the mouse lethality assay is provided in appendix 8.3. As SOPs are updated on a routine basis, the version supplied in this appendix may not necessarily be used throughout for the conduct of these experiments.

6.0 ANALYTICAL AND STATISTICAL PLAN

6.1 Sample Size

Experiments are designed so that the 95 percent confidence interval for the LD_{50} has a half width of 40 percent. To meet this objective, experiments are conducted using up to 30 animals in three stages for each serotype and treatment group. If after three stages the half width of the confidence interval is greater than 50 percent, an additional 10 animals are dosed. The half width of the confidence interval depends on variability in the estimated LD_{50} , which is a function of the number of animals dosed, slope of the dose-response curve, and the allocation of animals to the dose-response relationship. As the slopes are not yet known for the serotypes for the selected route of administration, results from Phase 2 of the MREF Task 96-45 were used to estimate the slope of the toxin dose-14 day lethality rate relations for serotypes A-E. The slopes ranged from 3.2 for serotype E to 7.75 for serotype B. The average slope of five was used for the sample size calculation. Fewer animals are required if the slope is larger than five. If the slope is 3.5, then 40 animals are required to produce a 50 percent confidence interval.

The spacing and number of dose groups for the mouse potency and mouse neutralization assays are based on data collected during Task 96-45.

6.2 Statistical Methods

For LD_{50} determinations in PBIG-, BBIG-, and CIG-treated guinea pigs, experiments are conducted using a stage-wise approach (Feder et al., 1991a and Feder et al., 1992). For each serotype, all available information and data are used to select the doses for the initial range finding. Probit dose-response models are fitted to dose-lethality data for both guinea pigs and mice using the method of maximum likelihood (Finney, 1971 and Feder et al., 1991b). Estimated parameters of probit dose-response models are used to compute LD_{50} values. Fieller's method (Finney, 1971) or delta method (Nelson, 1982) are used to compute a 95 percent confidence interval for the LD_{50} . For

each serotype, the 95 percent confidence interval for the LD_{50} will be computed after 30 guinea pigs have been exposed. If the half-width of the confidence interval, as defined in Section 6.1, is greater than 50 percent then 10 additional guinea pigs will be tested.

For each serotype, the protective ratio, defined as the LD_{50} for PBIG or BBIG treated guinea pigs divided by that for CIG-treated guinea pigs, is estimated. T-tests are conducted at the 5 percent significance level to determine if the protective ratios are statistically different than 1. Standard errors of the protective ratios are estimated using the delta method.

For each serotype, time-to-death is statistically analyzed to determine if significant differences exist between test and control groups. Regression models appropriate for right censored data are fitted to the time-to-death data using Proc Lifereg in the SAS computing system with time-to-death as the response variable and log toxin dose as the independent variable. Time-to-death is assumed to be log-normally distributed. For animals that survive the observation period, time-to-death is recorded as 336 hr (14 days) and is treated as right-censored. T-tests are conducted at the five percent significance level to statistically compare the slopes of the regression models between the two routes of administration. For both treatments, mean and standard deviation of time-to-death is estimated at the toxin LD_{50} .

The following responses are recorded, as applicable, for each animal: appeared normal, ruffled fur, labored breathing, weak limbs, total paralysis, droopy eyelids, and death. The occurrence of each sign in each of the monitored time intervals is noted and stored in a Microsoft Access database. Because the presence of one sign may mask the ability to observe another sign, the statistical analysis of the clinical signs data will be performed on combinations of specific signs. For instance, death or total paralysis precludes the ability to observe weak limbs in a specific observation interval. Therefore, the combined response death/total paralysis/weak limbs may be statistically analyzed. The combined response is defined to occur within a given interval if any one of the signs occurred in the interval.

For each response, the following three endpoints are calculated: incidence, time to onset (time between toxin injection and first observation of sign), and duration. If none of the signs comprising a combined response are exhibited during the observation period and the animal lives throughout the observation period, then the duration is set equal to zero. For these animals, time to onset is set equal to 336 hr (14 days) and is treated as right censored. Durations are treated as right censored for both animals that exhibit the sign throughout the observation period and for animals that die prior to the end of the observation period.

For each serotype and response, Fisher's Exact Test is conducted at the five percent significance level to compare the incidence of the combined response between test and control treatments. These tests are performed using Proc Freq in SAS. For each serotype and response, time to onset and duration of are statistically analyzed to determine if significant differences exist between the two treatments. Regression models appropriate for right censored data are fitted to the data using Proc Lifereg in the SAS computing system with log toxin dose as the independent variable. Times to onset are assumed to be log-normally distributed and durations are assumed to have either a normal or log-normal distribution.

6.3 Missing Value Handling

Mice used for the lethality assay are segregated and isolated by experimental group. Mortalities are recorded as they occur. At the end of a four day observation period, surviving mice are tabulated. Mortalities plus remaining survivors must add up to the beginning total. If mice have escaped during the experiment, mortality are reported as a percentage of the total animals that are accounted for. If fifteen percent or more of the mice are not accounted for in an experimental lethality curve, the assay results will not be used and the experiment will be repeated.

6.4 Records to be Maintained

Records will include but not be limited to the following:

1. Preparation of reagents
2. Toxin administration
3. Potency assay results
4. Animal care data
5. Mortality data
6. Clinical observations
7. Analyses of feed and water
8. Test material description, analyses, preparation, and administration.

A draft final report is prepared within 30 days, if feasible, after completion of the

exposures and analyses of the data. The draft final report includes:

- a. Experimental design
- b. Animal selection criteria and husbandry
- c. Statistical analyses of data
- d. Discussions and conclusions
- e. Quality assurance statement
- f. Location of archival records

Following receipt of draft final report comments from the Joint Program Office for Biological Defense, a final report will be prepared within 30 work days, if feasible.

7.0 REFERENCES

- Cardella MA (1964). "Botulinum Toxoids." In K.H. Lewis and K. Cassel Jr. (ed.) Botulism. U.S.P.H.S., Cincinnati.
- CDC (1994). Summary of Notifiable Diseases, United States (1993). "In Morbidity and Mortality Weekly Report." pp 42 (53).
- Doellgast GJ, Triscott MX, Beard GA, Bottoms JD, Cheng T, Roh BH, Roman MG, Hall PA, and Brown E (1993). "Sensitive Enzyme-linked Immunosorbent Assay for Detection of Clostridium Botulinum Neurotoxins A, B, and E Using Signal Amplification via Enzyme-linked Coagulation Assay." *J. Clin. Microbiology* 31:2402.
- Doellgast GJ, Beard GA, Bottoms JD, Cheng T, Roh BH, Roman MG, Hall PA and Triscott MX (1994). "Enzyme-linked Immunosorbent Assay and Enzymed-linked Coagulation Assay for Detection of Clostridium Botulinum Neurotoxins A, B, and E and Solution-phase Complexes with Dual-label Antibodies." *J. Clin. Microbiology* 32:105.
- Ekong TAN, Mclellan K, and Sesardic D (1995). "Immunological Detection of Clostridium Botulinum Toxin Type A in Therapeutic Preparations." *J. Immun. Methods* 180:181.
- Feder, PI, Hobson DW, Olson CT, Joiner RL, and Matthews MC (1991a). Stagewise, Adaptive dose Allocation for Quantal Response Dose-Response Studies, *Neuroscience & Biobehavioral Reviews*, 15, pp 109-114.

Feder PI, Olson CT, Hobson DW, Matthews MC, Joiner RL (1991b). "Statistical Analysis of Dose-Response Experiments by Maximum Likelihood Analysis and Iteratively Reweighted Nonlinear Least Squares Regression Techniques." *Drug Information J.*, 25, pp 323-334.

Feder PI, Hobson DW, Olson CT, Joiner RL, Matthews MC (1992). "Statistical Design Considerations for Stagewise, Adaptive Dose Allocation in Dose-Response Studies, In Biopharmaceutical Sequential Statistical Applications," Peace, K.E., ed., Marcel Dekker, New York.

Finney DJ(1971). Probit Analysis, Third Edition, Cambridge University Press, Cambridge, England.

Franz DR, Pitt LM, Clayton MA, Hanes MA, and Rose KJ (1993). "Efficacy of Prophylactic and Therapeutic Administration of Antitoxin for Inhalation Botulism."

Botulism and Tetanus Neurotoxins. B.R. DasGupta Ed. Plenum Press, New York.
Gill DM (1982). "Bacterial Toxins: A Table of Lethal Amounts." *Microbiol. Rev.* 46:86.

Lamanna C and Carr CJ (1967). "The Botulinal, Tetanal, and Enterostaphylococcal Toxins: A review." *Clin. Pharmacol. Ther.* 8(2), pp 286-332.

Lewis GE and Metzger JF (1980). "Studies on the Prophylaxis and Treatment of Botulism." *Natural Toxins*, pp 601-606.

McNally RE, Morrison MB, Berndt JE, Fisher JE, Bo'Berry JT, Puckett V, and Simini NJ (1994). "Effectiveness of Medical Defense Interventions Against Predicted Battlefield Levels of Botulinum Toxin A." Vols. 1 &2. Report funded by the U.S. Army Medical Research and Development Command and compiled by Science Applications International Corporation, Joppa, MD.

Nelson WB (1982). "Applied Life Data Analysis," John Wiley & Sons, New York.

Pearce BL, Borodic GE, Johnson EA, First ER, and MacCallum R (1995). "The Median Paralysis Unit: A more Pharmacologically Relevant Unit of Biologic Activity for Botulinum Toxin." *Toxicon* 33:217.

Seigel LS (1988). "Human Immune Response to Botulinum Pentavalent (ABCDE) Toxoid Determined by a Neutralization Test and by an Enzyme-linked Immunosorbent Assay." *J. Clin. Microbiol.* 26:2351.

MREF Protocol 130
G155553A
Medical Research and
Evaluation Facility
Effective Date July 17, 1998
Page 33 of 33

Shone C, Appleton N, Wilton-Smith, Hambleton P, Mōdi N, Gatley S and Melling J (1986).
"In Vitro Assays for Botulinum Toxin and Antitoxins." *Dev. Biol. Stand.* 64:141.

Summary of protocol FY92-16: "Therapeutic Efficacy of Equine F(ab')₂ Antitoxin for
Inhalation Botulism in Rhesus Monkeys." Undated report from Toxicology, Pathology and
Veterinary Medicine Divisions of the U.S. Army Medical Research Institute of Infectious
Diseases, Fort Detrick, Frederick, MD.

8.0 APPENDICES

APPENDIX 8.1

INVESTIGATORS, FACILITIES, AND QUALIFICATIONS

Investigator & Technician Qualifications/Training: Animal procedures will include animal identification, injection, anesthesia, clinical observations, and euthanasia. All animal procedures will be performed by trained Battelle personnel. MREF Animal Technicians are thoroughly trained for performing the appropriate animal handling procedures used in this protocol. Documentation of training for all involved personnel will be on file in the MREF facility or at the Battelle Headquarters in Columbus.

Dr. Thomas Gelzleichter received a Ph.D. degree in Pharmacology and Toxicology and has specialized the last seven years as an inhalation toxicologist with substantial experience directing *in vivo* inhalation experiments. Dr. Melissa Myers received her Ph.D. degree in Medical Microbiology and Immunology with a specialty in microbial biochemistry. Experience includes extensive work with various etiologic agents, enzymatic assays and protein biochemistry. Dr. Robert Hunt is the MREF Staff Veterinarian. Dr. Hunt has over twenty two years experience in animal handling, manipulations, and scientific research.

Accreditation: On January 31, 1978, Battelle's Columbus Operation received full accreditation of its animal care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The Medical Research and Evaluation Facility is a part of the facilities granted full accreditation. Battelle was inspected by the USDA in May 1998. No deficiencies were noted and we were commended for our environmental enrichment program.

Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-R-021) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institute of Health (NIH) on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966, as amended.

Protocols of all experiments using animals are reviewed and approved by (1) the Battelle Institutional Animal Care and Use Committees (IACUC) and (2) the U.S. Army Animal Use and Review Office and (3) the Battelle Biosafety committee prior to initiation of the study. The proper care and use of animals in the conduct of research described in this protocol is the responsibility of the Study Veterinarian, Study Director, and the Battelle Medical Research and Evaluation Facility (MREF) Management.

APPENDIX 8.2

PHARMACEUTICAL INGREDIENTS AND QUALITY

INVESTIGATORS BROCHURE

PENTAVALENT (ABCDE) BOTULINUM IMMUNE GLOBULIN (HUMAN)

Product Description:

Between 1978 and 1981, volunteers immunized with Pentavalent Botulinum Toxoid (A-E) at Fort Detrick and Pine Bluff Arsenal contributed immune plasma which was used to produce Botulism Immune Globulin. The plasma was fractionated by procedures described in BB-IND 1332 and summarized below.

Plasma Pool
Addition of Silicon Dioxide
Removal of SiO_2 by Centrifugation
Sterilization by 0.2 Micron Filtration
Concentration and Buffer Exchange
Anion Exchange Chromatography
Concentration and Buffer Exchange
Sterilization by 0.2 Micron Filtration
Bottling under N_2
Storage at -20 Degrees Centigrade

Botulism Immune Globulin has been tested in Phase I safety and pharmacokinetic studies administered by intravenous and intramuscular routes.

Currently, Lot 1A of this product is available for intravenous use. This lot was sterile filtered and bottled at 10 mL/vial in normal saline at a pH of 6.7-7.4. It contains no preservatives. For use it should be diluted to 15 mg/mL with sodium chloride for injection, U.S.P. The latest testing of Lot 1B demonstrated the following parameters (antibody titers will be reestablished by Battelle prior to initiation of study).

ITEM	RESULT
Serotype A Titer	201 IU/mL
Serotype B Titer	>9 IU/mL
Serotype C Titer	> 72 IU/mL
Serotype D Tit	> 152 IU/mL
Serotype E Titer	> 67 IU/mL
Protein Concentration	108 mg/mL
IgG Monomer and Dimer Percent	91 Percent

Description of Botulism:

Botulism is an uncommon, but frequently fatal, neuromuscular illness caused by the action of protein neurotoxins elaborated by *Clostridium botulinum*, and rarely, other clostridia. The organism is a gram-positive, obligately anaerobic, spore-forming bacillus. Typically, illness

usually follows ingestion of toxin from improperly preserved foods, but it can follow the ingestion of organisms or spores (infants), or the infection of a wound with *C. botulinum*, with ensuing *in vivo* toxin production. Importantly, inhalation toxicology studies in animals indicate that botulinum toxins have the potential to be used as biological warfare agents.

C. botulinum is widely distributed in soil and aquatic environments throughout the world. Seven antigenically distinct neurotoxin types of *C. botulinum* are recognized. The protein neurotoxin binds at the presynaptic membrane of neurons at peripheral synapses, leading to inhibition of acetylcholine release and neurotransmission blockade. The blockade is most evident clinically in the cholinergic nervous system and at the neuromuscular junction.

Hours to days following exposure to toxin, a neurologic disorder characterized by generalized weakness, lassitude, and dizziness develops. Diminished salivation with extreme dryness of the mouth and throat may cause complaints of sore throat. Urinary retention or ileus may also occur. Motor symptoms are usually present early in the disease; cranial nerves are affected first, with blurred vision, diplopia, ptosis, and photophobia. Bulbar nerve dysfunction causes dysarthria, dysphonia, and dysphagia. This is followed by a symmetrical, descending, progressive weakness of the extremities along with weakness of the respiratory muscles. Development of respiratory failure may be abrupt.

Recognized cases of botulism prior to 1950 had a mortality of 60 percent. In the early 1970s, the case fatality rate was approximately 23 percent. Therapy consists of administration of botulinum antitoxins (principally equine trivalent - A, B, and E), administration of antibiotics (for food and wound botulism), and intensive supportive therapy. With tracheostomy and ventilatory assistance, fatalities should be < 5 percent.

Previous Human Experience:

During 1980 five units of immune plasma (human) were administered iv to five normal volunteers. No adverse clinical responses occurred.

During 1992 eight volunteers received 10 mL of lot 2A; 4 intravenous and 4 intramuscular. There were no side effects after administration and the eight volunteers remained clinically healthy during the 6-month follow-up period.

In 1993 Lots 1B, 2B, and 1A were tested in four volunteers each as a single dose of 10 mL (15 mg/kg) intravenously. Initially two volunteers each received Lot 1B or 2B via 20 min slow-push. Dyspnea, facial flushing and dizziness developed in both volunteers receiving Lot 1B. These reactions resolved within a couple of minutes after administration was complete. Subsequently, one of the volunteers developed a fever of 101.2 which resolved the next day without medication or sequelae. One volunteer receiving lot 2B experienced dyspnea, facial flushing, and dizziness which resolved within 5 min after administration was complete. The volunteer subsequently developed abdominal cramps and nausea that was relieved after vomiting

with no sequelae. The protocol was modified so that the immune globulin was diluted with 59 mL of sterile saline and administered by IV pump over 1 hr. One volunteer who received Lot 1B developed a fever of 102 with shaking, chills and headache. This resolved with one dose of 975 mg Tylenol. There were no further sequela.

In 1994/95, four volunteers received Botulism Immune Globulin (Human) Lot 1A administered by intravenous route. Of the four volunteers entered into and completing this study, one volunteer developed a mild headache for 15 min during the one hour administration of Lot 1A immune globulin.

Three children suspected of having infant botulism have received this product between 1988 and 1995. All recovered with no reported adverse reactions or sequela.

Clinical Procedures:

1. An intravenous drip with normal saline should be started in the recipient.
2. The immune globulin (supplied as 10 mL/vial) should be diluted with 59 mL of sodium chloride for injection, U.S.P. (To 15 mg/mL) and administered intravenously over one (1) hour using the previously placed IV line and an IV pump. Additional doses may be administered as clinically indicated (i.e., transient benefit; disease progression).

Note 1: Emergency medical supplies and resuscitative equipment will be available in the immediate area for the treatment of any acute anaphylactic reaction should it occur.

Note 2: Adverse effects have been seen if this product is administered faster than the recommended rate.

Note 3: Rate of administration should be adjusted appropriately for children and infants.

3. Vital signs (heart rate, respiratory rate, temperature, and blood pressure) should be monitored at least every 15 min for 2 hr, beginning with initiation of the infusion. Vital signs should be continued to be monitored at least every four hours through the first 24 hr period.
4. Additional supportive care should be given as required.

Precautions:

1. Pentavalent Botulinum Immune Globulin (Human) is not licensed for general use and is distributed as an Investigational New Drug in accordance with the requirements of the U.S. Food and Drug Administration. The product must be administered under the supervision of qualified medical personnel.
2. The product could be administered to any individual suspected of having sustained an exposure to botulinum toxins by any route (food borne, wound infection, or inhalation).
3. There is no experience with the use of this product in pregnant patients. Attempts will be made to obtain a pregnancy test (serum bHCG or urine test, if serum test is unavailable) in female recipients of child bearing potential of this product. Information obtained from this test will be used for analytical purposes only, however, as pregnancy is not considered a contraindication for receipt of this immune globulin as therapy for suspected botulism. The potential benefit to a pregnant female of treating botulism with this product is felt to outweigh the potential risk of adverse consequence that she and/or her fetus might suffer as a result of having received the immune globulin.

Risks:

The risks associated with the immune globulin include the possibility of allergic reaction including fever, chills, and an uncomfortable sensation). Also IV administration may produce a drop in your blood pressure and difficulty breathing. In such a case, standard medical techniques would be used to treat those reactions. In addition, reasonable precautions are taken to minimize these risks. There is the added risk of transmission of an as yet unidentified agent in the immunoglobulin product. However, the product (Lot 1A) has been tested and found negative for hepatitis B surface antigen, but found to be surface antibody positive. This lot is negative for Hepatitis C when tested by PCR for hepatitis C DNA AR (National Institute of Health). Lot 1A is also negative for HIV-2 antibody and HIV-1 antigen by Western blot analysis, and radio immune precipitation for HIV-1 antigen.

APPENDIX 8.3

STANDARD OPERATING PROCEDURE FOR THE DETECTION AND QUANTITATION OF BOTULINUM TOXINS AND ANTITOXINS (ANTIBODIES) IN THE JM-1 BIOFACILITY AT THE MEDICAL RESEARCH AND EVALUATION FACILITY (MREF)

Manual Number:

Battelle SOP Number: MREF. X-014-04

Page 1 of 11

**STANDARD OPERATING PROCEDURE (SOP) FOR THE DETECTION AND
QUANTITATION OF BOTULINUM TOXINS AND ANTITOXINS (ANTIBODIES)**

Originated by: Melissa A. Myers
Melissa A. Myers, Ph.D.

Date 5/8/98

Reviewed by: David L. Stitcher
David L. Stitcher, CIH
MREF Environment, Safety and Health Officer

Date 5-8-98

Approved by: James E. Estep
James E. Estep, D.V.M., Ph.D.
Manager
Mepical Research and Evaluation Facility

Date 5-11-98

Reviewed and Registered by QAU:

Elisha N. Morrison
Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist

Date 5/11/98

Battelle's Medical Research and
Evaluation Facility (MREF)
505 King Avenue, JM-3
Columbus, Ohio 43201-2693

BATTELLE COPY

Manual Number:

Battelle SOP Number: MREF. X-014-04

Page 2 of 11

I. Scope

This SOP describes the general methods for the detection, quantitation and determination of potency of botulinum toxins and their antibodies.

II. Purpose

The anaerobic bacterium *Clostridium botulinum* produces exotoxins which act on the peripheral cholinergic nervous system. There are seven different serotypes of toxin (A, B, C, D, E, F, G) each produced by a different strain of the bacterium. Using the mouse bioassay, the potency of the toxin can be determined and the amounts of toxin-specific neutralizing antibodies can be measured.

III. References

- A. FSP SOP BIO-005, Safe Handling and Storage of Etiological Agents.
- B. SOP MREF. X-013, Intramuscular, Intraperitoneal and Subcutaneous Injections of Etiological Agents and Vaccines into Vertebrate Laboratory Animals.
- C. Material Safety Data Sheets (MSDS) for Botulinum toxins.
- D. Kautter, D. A., Solomon, H.M., and Rhosehamel, E.J., (1992) FDA Bacteriological Manual. 7th Ed.
- E. Ryan, T.P. (1989), Statistical Methods for Quality Improvement. John Wiley and Sons, New York.
- F. SAS (1995), SAS/QC Software: Usage and Reference. SAS Institute Inc., Cary, N.C.
- G. Method Number 11/Microbiology, Method for the preparations and storage of botulinum toxins.
- H. Method Number 13/Microbiology, Method for the detection and quantitation of proteins using the BCA protein assay..

BATTELLE

Manual Number:

Battelle SOP Number: MREF. X-014-04

Page 3 of 11

IV. Definitions

- A. LD₅₀ - the quantity of toxin that causes the death of half the animals dosed within a given time frame.
- B. MIPLD₅₀ Unit - the quantity of toxin which when injected intraperitoneally into mice causes death of half the animals within ninety-six hours. One unit of toxin is the LD₅₀. Botulinum toxins are quantitated by their potency or activity, not weight.
- C. Millipore water - water purified using the Milli-RO Plus[®] polished with the Milli-Q PF Plus[®] system (Millipore, Bedford, MA).
- D. TPCK - L-tosylamide-2-phenylethyl chloromethyl ketone - agent used to reduce the chymotrypsin contamination of trypsin.
- E. ED₅₀ - the amount of antitoxin/antibodies that protects half of the animals dosed from a predetermined toxin challenge dose.

V. Procedures

- A. Needed Materials. Botulinum toxins, antitoxins, antitoxin standards [World Health Organization (WHO) Antitoxin Standards. PerImmune, Inc. (PI) Antitoxin Standards], syringes, needles, mice, sterile gel phosphate buffer, test tubes, pipets and tips, vortex, centrifuge, centrifugal concentrators, pH meter, conductivity meter, trypsin, dibasic sodium phosphate, sodium chloride, hydrochloric acid (HCl), filter sterilizing flasks, vacuum pump, bovine serum albumin (BSA), and gloves.
- B. Hazard Information. Botulinum toxins attack the presynaptic terminal of the peripheral nerves, thereby blocking the release of acetylcholine and preventing muscle contractions. The botulinum toxins are among the most potent of toxins. An estimated lethal dose for humans is about 1 μ g by ingestion and 1 ng/kg by injection (assuming humans are at least as sensitive as mice). For mice, aerosol doses of botulinum toxins are typically 20-80 fold less toxic than injections. It is the responsibility of the users to read and understand the MSDS before handling botulinum toxins.
- C. Maintain all documentation in the appropriate study file.
- D. Preparation of Solutions (all volumes and amounts may be proportionately increased or decreased, as needed).

Manual Number:

BATTELLE COPY

Battelle SOP Number: MREF. X-014-04

Page 4 of 11

1. Sterile gel phosphate buffer - dissolve 2 g of gelatin and 4 g of dibasic sodium phosphate in 900 mL of millipore water. Heat the solution until the gelatin dissolves and then cool to room temperature. Adjust the pH to approximately 6.2 with HCl and bring the volume to 1 L. Aliquot into smaller volumes and autoclave for ~30 min. Cool and store refrigerated for up to 6 months. Document preparations on Form Number MREF Microbio.frm-100.
 2. Trypsin stock solution (10 mg/mL) - dissolve 10 mg of trypsin (TPCK-treated) in one mL of gel phosphate buffer. Prepare the solution just prior to use. Document preparation on Form Number MREF Microbio.frm-095.
 3. Phosphate buffered saline (PBS), 50 mM sodium phosphate, 0.2 M NaCl - dissolve 5.7 g of sodium phosphate monobasic (NaH_2PO_4), 1.3 g of sodium phosphate dibasic (Na_2HPO_4) and 11.0 g of NaCl in approximately 900 mL of millipore water. Check the pH of the solution. If the pH is outside the range of 5.9 - 6.3, discard and remake it. Adjust the pH to approximately 6.2 with NaOH or HCl. Bring the volume to one liter. Aliquot into the desired volumes and autoclave for at least 20 minutes. Store the solution refrigerated for up to six months. Document preparation on Form Number MREF Microbio.frm-067.
 4. Bovine serum albumin (BSA) solution (50 mg/mL) - dissolve 5.0 g of BSA in 100 mL of millipore water and filter sterilize with a 0.22 μm filter. Store the solution at less than -20 degrees C for up to 6 months. Thaw just prior to use. Document preparation on Form Number MREF Microbio.frm-097.
- E. Preparation of Toxin [**WARNING:** Botulinum toxins are extremely dangerous. Perform all manipulations in Class II or III Biological Safety Cabinet (BSC), unless specifically stated otherwise. Wear gloves at all times and take extreme care when using a needle and syringe for injecting animals]. Specific procedures for toxin preparation are found in reference III.G.
1. The botulinum toxins are received as ammonium sulfate precipitates in one batch for each individual serotype. Centrifuge the solutions to a pellet in a fixed angle rotor for approximately 15 min. Remove and discard the supernatant (treat as contaminated waste). The supernatant may be tested for potency using the LD_{50} assay, if desired.
 2. Dissolve the pellet in sterile PBS. Remove the excess ammonium sulfate by centrifugal concentration and reconstitution until the conductivity of the sample diluted to 1/100 with millipore water is within ~20 percent of the conductivity of the reconstitution buffer diluted 1/100 with millipore water, or until seven

BATTELLE COP

Manual Number:

Battelle SOP Number: MREF. X-014-04

Page 5 of 11

concentrating spins are completed.

3. Record the toxin preparation and aliquotting on Form Number MREF Microbio.frm -113.

F. Determination of MIPLD₅₀ Units (potency).

1. Following the dilution sheet (Form Number MREF Microbio.frm-100), set up multiple graded dilutions (using sterile gel phosphate buffer) of the botulinum toxin with the middle concentration containing an estimated 1 MIPLD₅₀ unit per inoculum. Determine the dilution factor based on the need of the particular experiment. For example, the initial potency determination is performed using a broad spectrum dilution series (e.g., 4-fold), in order to determine a more narrow range, while particular experiments may require a more compacted series (e.g., determination of aerosolized toxin) for a more accurate potency determination. It may first be necessary to perform a preliminary experiment in order to narrow the dilution range.
2. Inject mice intraperitoneally (See III.B., above) with approximately 0.5 mL of toxin at each concentration. The number of mice injected per group is based on the required precision. Refer to validation results (MREF Task 39) to determine the level of precision that is attained with differing numbers of mice.
3. Record the number of dead mice as well as live mice per group during daily clinical observations for a period of four days and at 96 ± 2 hr, or as specified in the study protocol. For each observation, if the total number of mice is different than the previous observation period, document this on the mouse observation form (Form Number MREF BL3.frm-021).
4. Determine the LD₅₀ dose based on the number of mouse deaths after 96 ± 2 hours. Determine the MIPLD₅₀ unit per mL in using probit analysis.
5. Depending on the strain of *C. botulinum*, some toxins such as botulinum E and sometimes C and D are excreted from the bacterial cell as un-nicked (inactive) toxins and require proteolytic treatment with trypsin prior to their use in bioassays. For toxins isolated from these strains of *C. botulinum*, use the following procedures.
 - a. Determine the protein concentration of the botulinum toxin using colorimetric or spectrophotometric procedures (See reference III.H.).

BATTELLE COPY

Manual Number: 46

Battelle SOP Number: MREF. X-014-04

Page 6 of 11

- b. Adjust the total protein content of the toxin to approximately 0.5 mg/mL (e.g., a 1 mL toxin sample with a protein concentration of 100 μ g/mL would require an addition of 400 μ g of protein or 8 μ L of the BSA solution).
- c. To the protein adjusted toxin sample, add 10 μ L of the trypsin solution (add 50 μ L of the trypsin to a 5 mL toxin sample, etc.).
- d. Incubate the toxin/trypsin/BSA solution at approximately 37 degrees C for approximately one hour.
- e. Once incubation is complete, dilute the sample for the assay or place on ice until ready to use.
- f. Determine the MIPLD₅₀ units. Use treated toxins for all subsequent assays and experiments.
- g. Record steps b through e on the dilution sheet (Form Number MREF Microbio.frm-100).

G. Determination of the Antibody (antitoxin) Concentrations.

1. Prepare a solution in gel phosphate buffer of standard botulinum toxin, so that one mL aliquots contain the predetermined toxin challenge dose. Toxin concentrations are expressed as a dilution factor of the 2A toxin preparations (A-0.00023, B-0.000040, C-0.0035, D-0.00024, E-0.00013).
2. Prepare a series of graded dilutions of the antibodies or antitoxins (see Table 1 for the midpoint for the current standards of use) in one mL volumes. Add one mL of the toxin challenge dose and mix, taking care not to generate foam (if more than 2 mL total volume is needed to dose the mice, a larger volume of antitoxin/antibodies is prepared and challenged with an equal volume of toxin challenge dose (i.e., 1.5 mL of antitoxin/antibody is combined with 1.5 mL of toxin challenge dose). Document this on the dilution sheets (Form Number MREF Microbio.frm-100).
3. Allow the antigen/antibody mixtures to stand for 60-120 min at room temperature.
4. Inject a dose of the mixture (~0.2 mL) intraperitoneally into mice (at least 3 mice per concentration). The number of mice dosed per group is based on the required precision. Refer to validation results (MREF Task 39) to determine the level of precision that is attained with differing numbers of mice.

BATTELLE COPY

Manual Number: 46

Battelle SOP Number: MREF. X-014-04

Page 7 of 11

5. Record the number of dead mice as well as live mice per group during daily clinical observations as described in paragraph V.E.3.
6. The end point of the titration is based on the death or survival of the mice after 96 ± 2 hr. Calculate the results using probit analysis.

H. Quality Control.

1. All Assays.

- a. Weigh the mice and assign them to a study. On the day of the study, mice must weigh 18.0-22.0 g for MIPLD₅₀ assays and 17.0-23.0 g for neutralization assays.
- b. Once a group of study mice is assembled, mice are chosen in an arbitrary fashion, assigned to a treatment group, injected, and placed in their home cage.
- c. During an experiment, open only one cage at a given time to ensure animal mixing does not occur between cages.
- d. After injection and before returning to the appropriate cage, visibly mark all animals to ensure that each animal is injected only once. If deemed necessary by the study protocol, mark the injection order of the animal.
- e. Once treated mice are placed into their home cage, do not remove them for any reason with the following exceptions: (1) Remove dead mice during daily observations; (2) In the rare event that a mouse needs to be removed and treated for non-botulinum related reasons, properly mark and identify it upon removal to ensure its return to the proper cage.
- f. If animals are randomized before use, take proper steps to ensure the identification of individual animals throughout the study, unless otherwise specified in the study protocol.
- g. Do not return escaped, unmarked animals to study.
- h. For the experiment to be valid, not more than 15 percent of the test mice are removed from the study for non botulinum reasons (e.g., escaped mice, injured mice, etc.) during the experiment.

BATTELLE COPY

Manual Number: 46

Battelle SOP Number: MREF. X-014-04

Page 8 of 11

2. MIPLD₅₀ Assays.

- a. Perform a buffer-only (BC) negative control with each set of experiments to show that the buffer has no ill effects on the mice.
- b. For toxin treated with trypsin, perform a buffer/trypsin/ BSA control (TC) to show this mixture has no ill effects on the mice.
- c. Use only mice weighing between 18.0-22.0 g for LD₅₀ assays.
- d. For each set of experiments, as a positive control to show that the toxin is potent, one group of mice receives doses containing approximately 20 MIPLD₅₀ units of toxin, or alternatively, a standard curve of serially diluted stock solutions is performed.
- e. At a minimum, one experimental group must have 50 percent or greater survival and one group 50 percent or greater mortality for the experimental results to be accepted (i.e., the experimental curve must touch or span the 50 percent mortality level).

3. Antibody detection.

- a. For each experiment, perform a standard curve using either serially diluted WHO or PI antitoxin standards, or alternatively, standards calibrated with the WHO or PI antitoxins.
- b. For the test to be valid, the observed LD₅₀ of the standard curve cannot vary more than 2 dilution units from the expected LD₅₀.
- c. For the test curve to be acceptable, there must be less than 50 percent lethality at the highest concentration of test serum (or antitoxin, in the case of the standard curve) and more than 50 percent lethality at the lowest concentration of test serum. In some cases (e.g., negative control serum, clinical samples), it is acceptable for all of the mice to either survive or die. In these cases, the Study Director determines if the test curve must be repeated. For experiments that are not repeated, express the results as less than or greater than the limits of quantitation for the assay.

BATTELLE COPY

Manual Number:

Battelle SOP Number: MREF. X-014-04

Page 9 of 11

- d. Perform a toxin only control (TOC) at the same concentration as the challenge dose for each set of experiments in order to show that the toxin is potent. Perform a BC negative control for each set of experiments.
- e. Use only mice weighing between 17.0-23.0 g for neutralization assays.
- f. As the toxins may slowly degrade over time, the dilution factors for the toxin challenge doses can be periodically reoptimized by titration against a fixed antitoxin amount (standard curve midpoint: see Table 1). If ED_{50} values begin to show a clear pattern of deviation from the expected value (see section V.H.3.h. below), new dilution factors are calculated by constructing titration curves in at least triplicate (on three separate days) and averaging the results to determine the new dilution factors. Once established, modify the SOP to reflect the new values. In the interim period, place a memo in any affected studies detailing the altered dilution factors.
- g. Toxin challenge doses (Table A) are reoptimized at a minimum every six months ($\pm$ one month) for all serotypes (see V.H.3.f). This period may be extended if the assay is not in use for a given serotype; however, reoptimization must occur before the assay is used to generate study data. Included in these experiments is a determination of toxin potency. If the challenge dose (in $MIPLD_{50}$) decreases substantially from historical levels, antibody standards are evaluated for loss of activity.
- h. A statistical process control method, CUSUM (See references III.E. and F.), is used to monitor assay variability. Parameters for the CUSUM method are determined and noted in a memo initiated by the statistical staff. An assessment of assay procedures will be initiated when the CUSUM method indicates the assay may be out of control. If the CUSUM method indicates reoptimization is necessary or if three out of six curves do not meet specifications, as outlined in Sections V.H.3.b. and c., the assay is not used until the reoptimization of the challenge dose has been completed.
- i. An estimate of assay variability is required for the CUSUM method. Historical data are used to estimate assay variability about the target midpoint for the standard curve. All available data are used for this estimate, except points where the assay is suspected to be out of control or points that are statistical outliers (determined by residual analysis). Variability estimates and CUSUM parameters may be adjusted when the assay is reoptimized periodically, or as needed, and changes noted in a memo initiated by the statistical staff.

Manual Number:

BATTELLE COPY

Battelle SOP Number: MREF. X-014-04

Page 10 of 11

I. Hazard Analysis.

1. An accidental needle stick while injecting a mouse is a route of toxin exposure for workers.
2. Simple manipulations (*e.g.*, opening the lid of a closed vessel, pipetting the solutions) cause aerosolization of toxins in solutions and could produce a toxin exposure.

J. Safety Precautions.

1. Anyone working with the botulinum toxins or at risk of toxin exposure must be vaccinated with the botulinum toxoid available from the Centers for Disease Control and Prevention (CDC) and produce a titer to botulinum toxin A. Vaccinated individuals have an estimated 250 times the protection of nonimmunized individuals for Bot A (*e.g.*, they are protected against 250 human LD₅₀ dose of botulinum toxin).
2. Perform toxin manipulations in either a Class II or III BSC, if working with concentrations >5000 MIPLD₅₀ units/mL (approximately one nonimmunized human LD₅₀ based on weight). Procedures performed outside a BSC (*e.g.*, intraperitoneal injections of toxin for LD₅₀ assays or neutralization assays as per reference III.C. above), must follow an approved Battelle SOP. Perform centrifugation only in sealed rotors. Once centrifugation is complete, remove the rotor from the centrifuge and open it in a Class II or III BSC.
3. Only trained personnel inject mice intraperitoneally with toxin for potency or antibody detection. Use extreme care since additional personal protective equipment (besides gloves) would reduce dexterity and may increase the risk of a needle stick. The maximum dose by accidental needle stick is estimated to be 100 MIPLD₅₀ of toxin, or 0.03 human LD₅₀ units (adjusting for the average weight of a human and assuming maximum injection volume of 0.1 mL), which is less than the protection level conferred by immunization.

BATTELLE COPY

Manual Number: 46

Battelle SOP Number: MREF. X-014-04
Page 11 of 11

TABLE 1

Standard Antitoxin	Lot Number	Challenge Dose Dilution Factor of Dilution 2A	Approximate Target Midpoint for Standard Curve (IU/mL)
WHO Bot B Antitoxin	13209 3000.1185	0.000040	0.0050
WHO Bot E Antitoxin	XII-141-2,500.9.3	0.00013	0.013
PI Bot A Antitoxin	PAAT03/07/96	0.00023	0.020
PI Bot C Antitoxin	PACT03/07/96	0.0035	0.012
PI Bot D Antitoxin	PADT03/29/96	0.00024	0.023

APPENDIX 8.4

**SUMMARY OF PROTOCOL FY92-16
THERAPEUTIC EFFICACY OF EQUINE F (AB')₂ ANTITOXIN
FOR INHALATION BOTULISM IN RHESUS MONKEYS**

- I. Objective. To evaluate therapeutic efficacy of equine F(ab')₂ antitoxin in monkeys exposed to an aerosol challenge of type A botulinum neurotoxin.
- II. Methods. A total of 20 monkeys were exposed to a highly lethal dose of type A botulinum neurotoxin by head only aerosol challenge. The challenge dose of botulinum toxin was 10,497 ± 4,025 (mean ± SD) mouse ip LD₅₀'s/kg. This was estimated to be 36 ± 13 monkey LD₅₀'s/kg. One group of monkeys was treated with F(ab')₂ botulinum antitoxin 24 hrs after aerosol challenge (n=8), while treatment was delayed in the second group until the onset of definitive clinical signs of botulism (n=8). The dose of antitoxin given at 24 hrs was either 14 IU/kg (0.1 human dose; n=4) or 143 IU/kg (1 human dose; n=4). In the experiment group treated at the onset of clinical signs, the dose of antitoxin was 14 IU/kg (n=2), 143 IU/kg (n=2), and 429 IU/kg (3 human doses; n=4). A control group was treated with 1 human dose of E. coli antitoxin at 24 hrs (n=2) or at the onset of first signs (n=2).
- III. Results. Overall results are summarized in Table 1 below. Data on individual monkeys is included in Table 2. The data from monkeys treated at the onset of clinical signs with either botulinum and E. coli antitoxin was also subjected to survival analysis (Figure 1).

TABLE 1. THERAPEUTIC EFFICIENCY OF EQUINE F(AB')₂ ANTITOXIN GIVEN 24 HR AEROSOL CHALLENGE OR AT THE ONSET OF CLINICAL SIGNS IN RHESUS MONKEYS CHALLENGED WITH BOTULINUM NEUROTOXIN SEROTYPE A

Treatment Group	Antitoxin	Inhaled BOT Monkey LD ₅₀ 's/kg	Survive/Total
E. Coli F(ab') ₂ 24 hrs		34-44	0/2
E. Coli F(ab') ₂ first signs		26-68	0/2
Bot F(ab') ₂ 24 hrs	14 IU/kg	6-43	4/4
Bot F(ab') ₂ 24 hrs	143 IU/kg	26-63	3/4
Bot F(ab') ₂ first signs	14 IU/kg	33-47	0/2
Bot F(ab') ₂ first signs	143 IU/kg	31-32	0/2
Bot F(ab') ₂ first signs	429 IU/kg	32-47	0/4

143 IU/kg = 1 human dose of antitoxin

14 IU/kg = 0.1 human dose of antitoxin

CONCLUSION: F(ab')₂ botulinum antitoxin given therapeutically 24 hrs after aerosol challenge of a highly lethal dose of type A botulinum toxin provided significant protection from lethality. Antitoxin alone did not protect against lethality if treatment was delayed until the onset of clinical signs, but did prolong survival time once clinical botulism was diagnosed. Time of treatment after aerosol exposure to botulinum toxin, rather than antitoxin dose, appears to be a critical determinant of botulinum antitoxin efficacy.

TABLE 2. THERAPEUTIC EFFICACY OF F(ab')₂ ANTITOXIN GIVEN 24 HRS AFTER AEROSOL CHALLENGE OR AT THE ONSET OF CLINICAL SIGNS IN RHESUS MONKEYS CHALLENGED WITH BOTULINUM NEUROTOXIN SEROTYPE A -INDIVIDUAL MONKEY DATA

Monkey No.	Treatment Group	Inhaled Bot (Monkey LD ₅₀ 's/kg)	Antitoxin	Survival Time After Antitoxin	Survival Time After Bot Aerosol Exposure
G927	E. Coli (Control)	34.0		28 hr	52 hr
88168	E. Coli	44.1		12 hr	36 hr
K427	E. Coli	26.4		9 hr	53 hr
G391	E. Coli	68.3		10 hr	40 hr
G279	bot F(ab') ₂	5.6	14	survived	survived
G401	bot F(ab') ₂	28.1	14	survived	survived
88167	bot F(ab') ₂	43.2	14	survived	survived
H592	bot F(ab') ₂	30.1	14	survived	survived
G240	bot F(ab') ₂	26.4	143	survived	survived
H739	bot F(ab') ₂	25.6	143	survived	survived
86277	bot F(ab') ₂	63.3	143	survived	survived
G902	bot F(ab') ₂	33.8	143	184 hr	208 hr
H401	bot F(ab') ₂	33.4	14	25 hr	54 hr
87162	bot F(ab') ₂	46.6	14	15 hr	60 hr
K520	bot F(ab') ₂	30.5	143	32 hr	96 hr
H813	bot F(ab') ₂	32.0	143	20 hr	66 hr
G658	bot F(ab') ₂	34.3	143	31 hr	66 hr
H499	bot F(ab') ₂	47.2	143	22 hr	57 hr
G419	bot F(ab') ₂	32.4	143	29 hr	61 hr
G201	bot F(ab') ₂	44.4	143	25 hr	54 hr

14 IU/kg = 0.1 human dose of antitoxin; 143 IU/kg = 1 human dose of antitoxin; 429 IU/kg = 3 human doses of antitoxin number in parenthesis in treatment group column is the time to the onset of clinical signs and thus time of antitoxin treatment.

17-MAR-57 16:28 FROM:OSAW 10

130

New Task 3

Protection of Guinea Pigs by Passive Immunization
with Pooled Human Botulinum Immune Globulin Obtained
30 Days Post Primary Series Immunization

- Objective: To demonstrate protection against botulinum toxin serotype A-E via passive transfer of pooled human botulinum immune globulin obtained from volunteers 30 days after immunization with the primary series of Pentavalent Botulinum Toxoid (A-E) vaccine.
- Rationale: This task will provide data to demonstrate the protective effect of antibodies elicited after the primary series immunizations. It will also allow a comparison of antibody protective effects for primary series antibodies obtained after boosters (the current Botulinum immune globulin-human).
- Methodology: Pooled sera obtained from volunteers after the primary series of immunizations with Pentavalent Botulinum Toxoid vaccine will be administered i.p. to guinea pigs to obtain a given circulating antibody level. Protection will be demonstrated by challenging groups of guinea pigs with each serotype of toxin (A-E).

Enclosure 3

**Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin
Obtained Following Vaccination**

MREF Protocol 130 (G155553A). Amendment No. 1

Effective date: 6/14/99

Change No. 1.

Section to be Changed: Study Director

The Study Director for this study is changed from Thomas Gelzleichter, Ph.D. to Robert Hunt, D.V.M.

Reason for Change:

Thomas Gelzleichter has left Battelle employment.

Impact on Study:

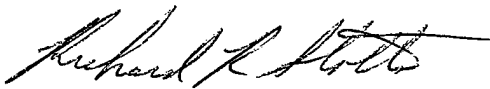
Validation experiments for antitoxin standards and reoptimization experiments have been conducted. However, the experimental phase of the protocol has not started. There is no impact on the study.

Approvals:



Robert Hunt, D.V.M.
Study Director

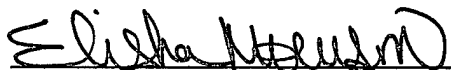
14 June 99
Date



LTC Richard R. Stotts, COR
USAMRICD

17 June 1999
Date

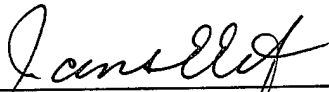
QA Registered:



Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist

6/28/99
Date

MREF Protocol 130
G155553A
Medical Research and
Evaluation Facility
June 14, 1999



James E. Estep, D.V.M., Ph.D.
Manager
Medical Research and Evaluation Facility

6-25-99

Date

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
October 4, 1999

**Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune
Globulin Obtained Following Vaccination**

MREF Protocol 130 Amendment No. 2

Change No. 1:

Reference MREF Protocol 130, page 14, 3.1. A., second sentence states "For PBIG plasma, samples will be screened by mouse neutralization assay before blending to eliminate nonresponders (i.e., those volunteers without detectable botulinum antibodies)."

Change to read: For PBIG plasma, corresponding serum samples from each individual in the study will be screened against all botulinum toxin serotypes using the mouse neutralization assay conducted as part of MREF Protocol 123. These assays will not be repeated under MREF Protocol 130. Mouse neutralization assay results will be reviewed prior to blending/purification. A minimum of approximately 15 liters of human plasma is required for the purification processing. If nonresponders (to any serotype) can be eliminated prior to blending, they will be eliminated. If plasma from nonresponders (to any serotype) is required in order to obtain the 15 liters of plasma required for the purification process, this plasma may be used."

Reason for change: Clarification. Serum samples are screened by the mouse neutralization assay as part of MREF Protocol 123, not plasma samples. The majority of the vaccinated volunteers generated antibodies (PBIG) against serotypes A, B, C, and D by day 113. However, a majority of the vaccinated volunteers did not generate detectable antibody titers against botulinum serotype E by day 113. In order to obtain the 15 liters of plasma required for the PBIG purification process, plasma from volunteers without detectable antibody against Bot E will be blended and purified.

Impact on the study: Undetermined as of this date. Failure to produce a detectable antibody titer against Bot E by day 113 postimmunization may be a "common" occurrence among immunized volunteers and thus may better represent the entire human population at that specific point in time.

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
October 4, 1999

Change No. 2:

Reference pg. 14, 3.1.B., second sentence states "Samples are assayed by the mouse neutralization assay before and after purification procedures to determine relative recovery of (1) neutralizing antibodies for serotypes A-E, (2) protein, (3) IgG, (4) IgM, and (5) IgA, and (6) IgG subtypes 1-4."

Reason for Change: Clarify the wording and intent of the protocol. As written, the statement is unclear.

Clarification: The statement should be two separate sentences and read as follows:

"Corresponding serum samples are assayed by the mouse neutralization assay (identified by patient number, under MREF Protocol 123) prior to the purification process.

After the purification process, samples will be assayed to determine relative concentrations of (1) neutralizing antibodies for serotypes A-E, (2) protein content, (3) IgG, (4) IgM, (5) IgA, and (6) IgG subtypes 1-4 for each lot of PBIG and BBIG."

Impact on Study: None. The mouse neutralization assay is the only assay essential to the screening of individual serum samples. The other assays do not provide any criteria relative to the characteristics of the purified product. These assays, if conducted prior to blending and purification, have no added value for the study.

Change Number 3:

Reference page 27, 4.1, Test and Control Articles, second sentence states "Prevaccination plasma is used to produce control article (PBIG)."

Change to read: Prevaccination plasma is used to produce control article (CIG).

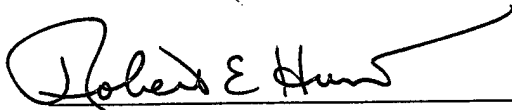
Reason for Change: Correction

Impact on the study: None.

Effective Date: October 4, 1999

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
October 4, 1999

Approved by:

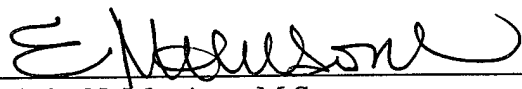

Robert E. Hunt, D.V.M.
Study Director

4 Oct 99
Date


Richard R. Stotts, LTC, VC
Contracting Officers Representative
U.S. Army

29 Oct 1999
Date

Quality assurance Review and Registration:


Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist

10/29/99
Date

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
February 16, 2000

**Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin
Obtained Following Vaccination**

MREF Protocol 130 Amendment No. 3

Change No. 1.

Reference page 9, Biological Agents / Test Articles, 2nd paragraph, 4th sentence, "Before use....by radial immunodiffusion (RID), relative abundance of IgG subclasses (1-4) will be determined by enzyme-linked immunosorbent assay (ELISA), and protein content will be determined by colorimetric assay."

Change to read:

"Before use on study IgG, IgM, IgA, IgG subclasses 1-4, and protein content will be determined."

Reason for Change:

Personnel changes over the last year have resulted in our laboratory not having the technical expertise to conduct these assays in-house in a cost efficient and timely manner.

Impact:

None. These assays will be subcontracted to LabCorp, a local laboratory that routinely performs analytical assays on human clinical samples. The assays are automated and will be performed under GLP guidelines and documented appropriately.

Effective Date: 16 February 2000

Change No. 2.

Reference page 15, 3.1.C (Avidity Determinations), 2nd sentence, "Avidity determinations will be conducted at L+/10, L+/33, and L+/100 doses for vaccine lots 003 and 004 (both PBIG and BBIG) and also for BIG."

Change to read:

"Avidity determinations will be conducted at L+/10, L+/33, and L+/100 on 2 lots (randomly designated A and B) of PBIG, pooled PBIG, and pooled BBIG."

Reason for Change:

Plasma samples derived from human volunteers by plasmaphoresis (Phase I / II Evaluation of Safety and Immunogenicity of Pentavalent Botulinum Toxoid (A-E) Administered to Healthy Volunteers) on day 211 were insufficient to generate 15 liters of either lot (designated A and B) of BBIG alone; thus, the available plasma samples (10 from each lot) were blended into a single lot of BBIG prior to purification. To compare PBIG avidity on day 112 against BBIG avidity on day 211, equal numbers of vials of purified PBIG A and PBIG B (Lots 003 and 004) will be pooled, at the same ratio as the day 211 pooled BBIG.

Impact:

None. The difference in BBIG avidity between lots 003 and 004 cannot be compared. The avidity of PBIG and BBIG will be compared by pooling the samples.

Effective Date: 16 February 2000

Change No. 3.

Reference page 15, D., "Determination of LD₅₀....", 3rd sentence, "Experiments are restricted to serotypes A, B, and E, and to the plasma pool yielding the highest neutralizing antibody levels (i.e., lot 003 or 004).

Change to read:

"Experiments will be performed in the order of preference serotypes A, C and D depending upon the availability of time and resources.

Reason for Change:

The study design allowed for 15 liters of human plasma to be purified from human volunteers for CIG, PBIGA, PBIGB, and BBIGA, and BBIGB. PBIG antibody titers to serotypes B and E are lower than expected and there are not sufficient quantities of

2/17/00
Date

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
February 16, 2000

purified human plasma available to conduct studies on either serotype B or E. The client has requested that we use the available purified human plasma to conduct efficacy studies on serotypes A and C. The contract has a finite life; however, depending upon the availability of resources, serotype D will also be tested.

Impact:

None. The study was designed with only a presumption of how much plasma might be available from the independent human volunteer study. The study design is being changed to reflect availability of human plasma collected from volunteers. A significant population of the human volunteers dropped-out of the immunization study prior to study completion (day 211 plasmaphoresis) and thus plasma available for purification and testing was much less than expected.

Effective Date: 16 February 2000

Approved By:

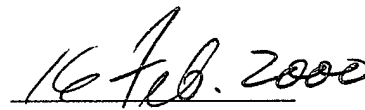
See Page 41

Robert E. Hunt, D.V.M.
Study Director

Date



David Steele, D.V.M.
Sponsor Representative, JVAP



Date

Reviewed and Registered By:

Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist

Date

**Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin
Obtained Following Vaccination**

MREF Protocol 130 Amendment No. 4.

Change No. 1.

Reference Amendment No. 3 Change 1 and page 15, 3.1.B., last sentence "Neutralizing antibody, Ig, and protein determinations are conducted as per Battelle SOP requirements".

Change to read:

"Neutralizing antibody concentration assays will be conducted as per Battelle SOP requirements. Ig and protein determinations will be conducted as per LabCorp SOP requirements.

Reason for Change:

The Ig and protein determinations have been subcontracted to LabCorp for analysis. Battelle SOP requirements do not apply to subcontractors (LabCorp).

Impact on Study:

None. This correction should have been addressed in Protocol Amendment 3, Change 1. The assays were conducted in accordance with GLP guidelines.

Effective Date: 16 February 2000.

Change No. 2.

Reference page 14, 3.1.A., last sentence, Plasma is segregated into five separate pools based on the lot of vaccine received.

1. Study day zero prebleed (prior to vaccination)
2. 4 weeks after primary series (vaccine lot -003)
3. 4 weeks after primary series (vaccine lot -004)
4. 4 weeks after 6 month boost (vaccine lots -003)
5. 4 weeks after 6 month boost (vaccine lot -004)

Change to read:

Plasma is segregated into five separate pools as indicated below:

1. Study day zero prebleed first lot (prior to vaccination)
2. Study day zero prebleed second lot (prior to vaccination)
3. 4 weeks after primary series (vaccine lot 003)
4. 4 weeks after primary series (vaccine lot 004)
5. 4 weeks after 6 month boost (vaccine lots 003 and 004 combined)

Reason for Change:

Clarification: The protocol study design was written to include five lots of immune globulin; one lot of prevaccination immunoglobulin (CIG) and two lots each of primary (PBIG) and two lots of booster (BBIG) immunoglobulin. Amendment 3, Change 2 addressed combining the BBIG into one lot. This amendment addresses the requirement for one additional lot of control immunoglobulin (CIG) to be purified in order to have enough of the control material to conduct the testing. Both lots of CIG will be tested the same and will have distinct lot numbers for identification purposes. Five lots of immune globulin will have been purified; two lots were control lots from nonimmunized volunteers, two lots were produced from PBIG plasma, and one lot was produced from BBIG plasma.

Impact on Study:

None: Reference Amendment 3, Change 2. The purification of an additional lot of CIG has no impact on the study.

Effective date: 16 February 2000

Change No. 3.

Reference Page 15, 3.1.C., 3rd sentence, "Avidity is determined for serotypes A-E."

Change to read:

Avidity is determined for serotypes A, C, and D, in that order and as resources permit.

Reason for Change:

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
March 7, 2000

Sufficient purified plasma is not available to conduct all the testing that was originally planned. The client has requested that serotypes A, C, and D be tested, in that order, and as resources allow for both the avidity and the protection studies (See Amendment 3, Change 3).

Impact on Study:

None.

Effective Date: 16 February 2000

Change No. 4.

Reference page 16, 3.1.D., Immune Globulin Pretreatment, 3rd sentence, "CIG dosages are normalized to the protein content of the BIG or BBIG dosages."

Change to read:

"CIG dosages are normalized to the protein content of the PBIG dosages."

Reason for Change:

BIG is a typographical error (it should have been PBIG) and since the PBIG protein content is higher than the BBIG protein content, CIG will be normalized to the PBIG.

Impact on Study:

None. The CIG is a control immunoglobulin and should not provide any protection against the toxin challenge. The CIG should be normalized to the highest protein content to insure the scientific validity of the study.

Effective Date: 6 March 2000

Change No. 5.

Reference page 26, 3.9.3, Safety Variables, Containment Level, "All animals that die or are euthanized are double bagged and autoclaved before removal from the laboratory facility."

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
March 7, 2000

Change to read:

All animals that die or are euthanized are double bagged, stored in a freezer if necessary, and incinerated in building JM-3."

Reason for change:

Clarification: Per Battelle SOPs, botulinum treated animals do not require containment housing and are not autoclaved unless they are housed in the BL-3 containment laboratory. Botulinum treated animals do not leave the facility, they are incinerated in building JM-3 per Battelle SOPs.

Impact on Study:

None.

Effective Date: 6 March 2000

Change No. 6.

Reference Page 14, 2.1, Primary Objectives, 1st sentence, "The objective of the proposed research is to determine the level of protection afforded by treatments with PBIG and BBIG in protecting guinea pigs against challenges with botulinum toxins A, B, C, D, and E".

Change to read:

"The objective of the proposed research is to determine the level of protection afforded by treatments with PBIG and BBIG in protecting guinea pigs against challenges with botulinum toxins A, C, and D, in that order, and as resources allow."

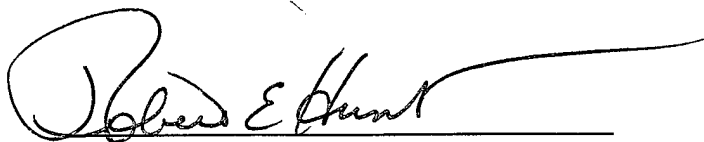
Impact on Study:

None. This change should have been addressed in Amendment 3, Change 3.

Effective Date: 6 March 2000

MREF Protocol 130
Study Number G155553A
Medical Research and
Evaluation Facility
March 7, 2000

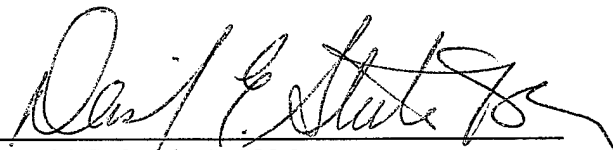
Approved By:



Robert E. Hunt, D.V.M.
Study Director

10 March 00

Date



David E. Steele, D.V.M.
Product Manager, JVAP

8 March 2000

Date

Quality Assurance Review and Registration:



Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist

3/10/00

Date

APPENDIX B

Study Deviations

MEDICAL RESEARCH AND EVALUATION FACILITY
DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G155553A

Record: Mouse Assay Weight Sheets Deviation No. (Assigned by QAU): DR-027^{JS}

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): MREF X-027

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):

9/9/98

Description of Deviation:

As stated in SOP MREF X-027, weight sheets will be printed out and signed on the day that the mice are weighed. Due to an oversight by the data recorder, the sheets were not signed until the day after the work was performed.

Cause of Deviation:

Technical oversight.

Impact of Deviation:

The sheets were printed out the day of weighing the mice. The technician weighed the mice, upon finishing, saved the sheets, and then printed them out. By opening the weight sheets back up, it can be verified that the sheets that were printed out correspond to those sheets that were saved from the day the mice were weighed. Therefore, there is no impact on the study.

Corrective Action: The technician was reminded of the importance of verifying and signing the weight sheets on the day that the work is performed.

CA No. Assigned by QAU (if applicable): NA

If deviation is planned, effective date:

Deviation form Prepared by/Date: Dana R. Pohlman 9-17-98

Deviation Reviewed and Corrective Action Accepted by/Date: Dr. R. Ziffer 9/18/98

Deviation Reviewed and Registered by QAU/Date: Editha Holledorn 9/22/98
JS 9/23/98

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G155553A

Record: Mouse Observations Deviation No. (Assigned by QAU): DR-028^{JS}

Type of Deviation

☐ GLP (Section): _____

☐ SOP (Number): _____

☒ Protocol (Number):

130

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):

8/27/98

Description of Deviation:

As stated in MREF Protocol 130, study animal observations will be performed and recorded twice daily. PM observations were not recorded on 8/27/98 for group E from experiment 7095.

Cause of Deviation:

Technical oversight.

Impact of Deviation:

There was no impact on the study considering that the mice were dosed earlier in the day. As seen in past experiments, it is highly unlikely that mice dosed earlier in a given day will die by the PM observation period. As data analysis is based only on a 96 hour analyses, there is no impact on the study.

Corrective Action: The technician was reminded of the importance of accurately and promptly recording data once observations are taken. The technician was also reminded to double check all observation sheets upon finishing to ensure that everything is properly documented. CA No. Assigned by QAU (if applicable):

DR 9-21-98

If deviation is planned, effective date:

Deviation form Prepared by/Date: Dana S. Bohman 9-18-98

Deviation Reviewed and Corrective Action Accepted by/Date: Jon S. Siffert 9/22/98

Deviation Reviewed and Registered by QAU/Date: Elisha Nelson 9/22/98
JS 9/23/98

Form No. MREF Facility-035-01 (Revised 4/6/98)

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs By Passive Immunization with pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G155553A

Record: Mouse Observations Deviation No. (Assigned by QAU): DR#34

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): MREF X-014

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s): 9/13/98

Description of Deviation:

As stated in SOP MREF X-014, mouse observations will be recorded 96 +/- two hours from the time that they are dosed. On 9/13/98, experiment numbers 7141 (groups A-C), 7144 (groups A-C), and 7150 (groups A, C-E) were observed and removed from study 1 minute to 37 minutes late.

Cause of Deviation:

Technical Oversight

Impact of Deviation:

There was no impact on the study because the mice were observed and taken off late. No mice were recorded as dying between the AM observation and the time that they were observed and removed from study. Also the latest that they were observed and removed was 37 minutes which is not a considerably long time from the time the final observation should have occurred.

Corrective Action:

CA No. Assigned by QAU (if applicable): NA

The technician was reminded of the importance of observing and removing mice from study in the given time frame. They were reminded to double check the times before observing and documenting the final observation and removal from study. JB 10/2/98

If deviation is planned, effective date:

Deviation form Prepared by/Date: Dana X Pollman 9-23-98

Deviation Reviewed and Corrective Action Accepted by/Date: Dr. Helfert 9/24/98

Deviation Reviewed and Registered by QAU/Date: Enthus 10/5/98 Study Director (if applicable) Jessica 10/5/98

MEDICAL RESEARCH AND EVALUATION FACILITY
DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin Obtained Following Immunization ☒ VACCINATION

Study Number(s): G155553A

Record: Rm. 117 Obs. Temperature, Humidity, Census, Daily Room Schedule, etc.

Deviation No. (Assigned by QAU): DR# 50

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): MREF IV-001-05

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s): September 3, 1998

Description of Deviation:

No temperature or relative humidity values were taken in room 117, in the morning of September, 3 1998.

Cause of Deviation:

Technician failed to obtain temperature and relative humidity readings.

Impact of Deviation:

None. There is a backup data system, using a Honeywell system. We are able to view historical data and can verify that temperature and relative humidity are in acceptable ranges.

Corrective Action:

CA No. Assigned by QAU (if applicable): NA

The technicians reviewed MREF IV-001-05, section V.C.1. They were also reminded of the importance to obtain the temperature and relative humidity readings twice per day.

Linda Baker- Linda Baker 10/8/98

Dana Pohlman- Dana Pohlman 10-8-98

If deviation is planned, effective date:

NA

Deviation form Prepared by/Date: James Mann Jr 10-7-98

Deviation Reviewed and Corrective Action Accepted by/Date: Jim Self 10/20/98

Deviation Reviewed and Registered by QAU/Date: ENW 11/17/98 Study Director (if applicable) JES 11/17/98

Form No. MREF Facility-035-01 (Revised 4/6/98)

ENW - Jfm 11-16-98

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs by Passive Immunization with Pooled Human Immune Globulin Obtained Following Vaccination(G155553A) and Evaluation of Test Material by Micronucleus Induction (C1016425A)

Study Number(s): G155553A and C1016425A

Record: Daily Room Activity Schedule Deviation No. (Assigned by

QAU): DR-105

Type of Deviation

☐ GLP (Section):

☒ SOP (Number): MREF VII-012

☐ Protocol (Number):

☐ Method (Number):

☐ Other:

Date of Deviation(s):

12/13/98

Description of Deviation:

Daily Room Activity Schedule did not indicate that the feed was checked. Technician did confirm that the feed was checked but erroneously not documented.

Cause of Deviation:

Technician oversight

Impact of Deviation:

None.

Corrective Action:

CA No. Assigned by QAU (if applicable):

NA

Technician was reminded to double-check all GLP documentation, at the time of performance, for correct and accurate recordkeeping.

QA 4-13-99

If deviation is planned, effective date:

Deviation form Prepared by/Date: Michael L. Clagett 4-12-99

Deviation Reviewed and Corrective Action Accepted by/Date:

Robert E. Hunt 13 April 99

Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date:

Elaine S. W. 4/16/99

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G155553A

Record: Rodent Mortality and Group Survival Data

Deviation No. (Assigned by QAU): DR-168

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): MREF X-014

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):

12/15/98

Description of Deviation:

SOP MREF X-014 states that mice will be taken off study 96 hours, +/- 2 hours from the dose time. Experiment #7190 Groups F - H were taken off of study 3 - 9 minutes too early.

Cause of Deviation:

Technician removing mice from study became inattentive to time restraints.

Impact of Deviation:

None.. Comparison of the number of animals alive during the AM observation and the end time shows no change. With a maximum time deviation of 9 minutes early it is unlikely that the final end point data for these experiments would have changed.

Corrective Action:

CA No. Assigned by QAU (if applicable): NA

The technician removing animals from study was reminded of the time constraint.

If deviation is planned, effective date:

NA

Deviation form Prepared by/Date: MC 9-16-99

Deviation Reviewed and Corrective Action Accepted by/Date:

Robert E Hunt / 17 Sept 99

Deviation Reviewed and Registered by QAU/Date:

E. H. Hunt / 11/3/99

Form No. MREF Facility-035-01 (Revised 4/6/98)

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: **Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination**

Study Number(s): **G155553A**

Record: **Refrigerator and Freezer Temperature Record**

Deviation No. (Assigned by QAU): **DR-181**

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): **MREF V I- 023**

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):

12/13/99

Description of Deviation:

Temperature for Freezer ID # 786650960 was not recorded.

Cause of Deviation:

Oversight by supervisor.

Impact of Deviation:

None. Temperatures recorded before and after this date confirm that the freezer was within range and working properly.

Corrective Action:

CA No. Assigned by QAU (if applicable): _____

Supervisor will attempt to be more attentive towards the proper recording of the freezer temperatures when the assigned technician is absent.

MC 1-4-00

If deviation is planned, effective date:

Deviation form Prepared by/Date: *Michelle R. Clagett 1-4-00*

Deviation Reviewed and Corrective Action Accepted by/Date: *Robert E. Hunt*
Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date: *J. Evans 1/17/00*

MEDICAL RESEARCH AND EVALUATION FACILITY
DEVIATION FORM

Study and/or Facility record affected

Study Title Protection of Guinea Pigs By Passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G15553A

Record: N/A

Deviation No. (Assigned by QAU): DR-197

Type of Deviation

☐ GLP (Section): _____

☐ SOP (Number): _____

☒ Protocol (Number): 130

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):

03/01/00

Description of Deviation:

MREF Protocol 130 page 24, paragraph 4 states that for each lot of purified immune globulin, the antibody content is determined before and after plasma processing using a single broad range finding curve followed by two narrow range curves (ran in duplicate on different days and averaged) for all five serotypes. The antibody content of each lot prior to purification was not determined.

Cause of Deviation:

Prior to the shipment of each lot, serum from each subject comprising the total lot was screened to determine the neutralizing antibody content. As per protocol requirements, only responders were included. This was deemed sufficient by the study director. A plasma sample of each combined lot prior to purification was not obtained and therefore the antibody content determination prior to purification cannot be determined.

Impact of Deviation:

None.

The approximate antibody content of each combined lot can be determined by taking the geometric mean of the antibody titers for all of the subjects included in the lot. Although the antibody content of each lot prior to purification may have been beneficial in determining the beginning broad range curves for antibody content determination after purification, the information garnered from the pre-purification antibody determination is not used for any dose calculations or determinations and has no impact on the operation or outcome of the study.

Corrective Action:

CA No. Assigned by QAU (if applicable): DA

All lots have been purified therefore no pre-purification antibody determination will be done.

If deviation is planned, effective date:

NA

Deviation form Prepared by/Date: Michelle L. Clagett 3-2-00

Deviation Reviewed and Corrective Action Accepted by/Date: Robert E. Hunt 2 MARCH 00

Deviation Reviewed and Registered by QAU/Date: E. M. [Signature] 3/6/00
Study Director (if applicable)

MEDICAL RESEARCH AND EVALUATION FACILITY
DEVIATION FORM

Study and/or Facility record affected

Study Title: **Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination**

Study Number(s): **G155553A**

Record: Animal Receipt Deviation No. (Assigned by QAU): **DR-200**

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): **VII-026-02**

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):

03/09/00(guinea pigs); All previous mouse shipments

Description of Deviation:

As stated in SOP VII-026-02, number and sex of animals will be checked as they are being caged to assure appropriate number and sex have been received. Animals were being sexed during restraint prior to dosing.

Cause of Deviation:

Technical misunderstanding of SOP requirements

Impact of Deviation: None

Guinea pigs and mice were verified to be male during restraint prior to dosing therefore all protocol requirements were still met.

Corrective Action: All future shipments of guinea pigs will have the sex verified upon receipt. Due to the voluminous amount of mice received per shipment and the additional labor involved in sexing mice upon receipt, an SOP change will be forthcoming to allow for flexibility in sex verification while still maintaining protocol compliance.

MC 3-23-00

If deviation is planned, effective date:

Deviation form Prepared by/Date: *Michelle L. Clagett 3-28-00*

Deviation Reviewed and Corrective Action Accepted by/Date: *Robert E. Hunt 28 March 00*

Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date: *Jessica Evans 4/11/00*

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G155553A

Record: _____ Deviation No. (Assigned by QAU): DR-1215

Type of Deviation

☐ GLP (Section): _____

☐ SOP (Number): _____

X Protocol (Number): 130

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s): 10 March thru 12 May, 2000

Description of Deviation: Study Director directed euthanasia of "moribund" guinea pigs to minimize pain and distress of botulinum intoxicated animals.

Cause of Deviation: Reference Protocol 130, page 26, 3.8, last sentence: "No treatment will be given for study related illness." and page 29, 6.2, 3rd paragraph: "For each serotype, time-to-death is statistically analyzed to determine if significant differences exist between test and control groups."
Study Director directed euthanasia of "moribund" guinea pigs to minimize pain and distress.

Impact of Deviation:

Minimal to None. Although time-to-death can not be statistically analyzed in this study, time-to-death was statistically analyzed in Task 97-52 and no significant differences were found. Although not planned, statistical analysis of time-to-euthanasia could be substituted for time-to-death without any significant impact on the integrity of the data. Both the Study Director and the Study Supervisor have considerable experience with botulinum toxin(s) in multiple animal species. Both individuals have the required expertise to correctly and appropriately identify a clinically "moribund" animal. In the experience of both, animals correctly identified as "moribund" due to a botulinum toxin challenge do not recover and death is imminent (prior to the next observation time). Pain and distress were minimized by euthanizing moribund animals.

Corrective Action: NA CA No. Assigned by QAU (if applicable): NA

If deviation is planned, effective date: 25 April 00 to 12 May 00

Deviation form Prepared by/Date: Robert E Hunt, DVM / 25 April 00

Deviation Reviewed and Corrective Action Accepted by/Date: Robert E Hunt, DVM / 25 Apr 00

Deviation Reviewed and Registered by QAU/Date: [Signature] 5/8/00
Study Director (if applicable)

Form No. MREF Facility-035-01 (Revised 4/6/98)

Don EMM 5/8/00

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs by Passive Immunization with Human Botulinum Immune Globulin Obtained Post Primary and Post Six-Month Immunization

Study Number(s): G155553A

Record: Deviation No. (Assigned by QAU): DR-237

Type of Deviation

☐ GLP (Section):

☒ SOP (Number): MREF SOP X-014

☐ Protocol (Number):

☐ Method (Number):

☐ Other:

Date of Deviation(s):

Study Date: 04/13/00

Description of Deviation:

Per MREF SOP X-014, a toxin only control group should be included in order to demonstrate the potency of the toxin.

Experiment 61004 did not include a Toxin only control (TOC).

Cause of Deviation:

An error in the dilution preparation for experiment 61004 Dose Group J (TOC) inadvertently occurred. This group was not dosed.

Impact of Deviation:

None. This experiment did not pass the assay acceptance criteria and was repeated. (See study date 04/27/00 for results that passed the acceptance criteria.)

Corrective Action:

CA No. Assigned by QAU (if applicable): NA

The person preparing the dilutions was reminded of the importance of adhering to the procedures outlined in the dilution sheets. 6/19/00

If deviation is planned, effective date:

NA

Deviation form Prepared by/Date: Rebekah A. Starnier 06/09/00 6/9/00

Deviation Reviewed and Corrective Action Accepted by/Date: Robert E. Hines 15 June 00

Deviation Reviewed and Registered by QAU/Date: E. Hines 8/22/00

Form No. MREF Facility-035-01 (Revised 4/6/98)

MEDICAL RESEARCH AND EVALUATION FACILITY
DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G155553A

Record: Rodent Mortality and Group Survival Data

Deviation No. (Assigned by QAU): DR-247

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): MREF X-014

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):

12/13/99

Description of Deviation:

SOP MREF X-014 states that all mouse neutralization assays will be injected into mice at 60 – 120 minutes after the addition of toxin to the antibody/antitoxin. The mice in experiment CIG30004 and PBA30002 Groups A – D were dosed at 49-56 minutes after the addition of toxin.

Cause of Deviation:

Wrong dosing team inadvertently dosed these experiments using the wrong time schedule.

Impact of Deviation:

None. The dosed time of a maximum of 10 minutes prior to the allotted time represents less than a 6% decrease of the minimum time.

Corrective Action:

CA No. Assigned by QAU (if applicable): NA

Dosing team was informed of this mistake and was reminded to pay more strict attention to the experiments numbers.

MC 8-22-00 ES 8-24-00

If deviation is planned, effective date:

NA

Deviation form Prepared by/Date: Michelle L. Clagett / 08/22/00

MC 8/22/00

Deviation Reviewed and Corrective Action Accepted by/Date:

Robert E. Hume 22 Aug 00
Study Director (if applicable)

Deviation Reviewed and Registered by QAU/Date:

ERH 10/9/00

MEDICAL RESEARCH AND EVALUATION FACILITY
DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guinea Pigs By passive Immunization With Pooled Human Immune Globulin Obtained Following Vaccination

Study Number(s): G155553A

Record: Rodent Mortality and Group Survival Data

Deviation No. (Assigned by QAU): DR-248

Type of Deviation

☐ GLP (Section): _____

☒ SOP (Number): MREF X-014

☐ Protocol (Number): _____

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s):
02/22/00

Description of Deviation:

SOP MREF X-014 states that all mouse neutralization assays will be injected into mice at 60 – 120 minutes after the addition of toxin to the antibody/antitoxin. The mice in experiment BBG 10006 Groups G – I were dosed 122-129 minutes after the addition of toxin

Cause of Deviation:

Not enough time scheduled between experiments.

Impact of Deviation:

The dosed time of 129 minutes, at the most, past the allotted time represents only an increase of approximately 7% of the maximum time. Based on historical analysis, the integrity of the dilution has shown no deterioration over the one hour time period. Seeing that this deviation is, at the most, only 7% from the allotted time period, it is highly unlikely that the experimental results were affected.

Corrective Action:

CA No. Assigned by QAU (if applicable): NA

More time will be allotted between experiments when planning future experiments.

If deviation is planned, effective date:

NA

Deviation form Prepared by/Date: Michelle L Clagett 8/22/00

Deviation Reviewed and Corrective Action Accepted by/Date: Robert E. Hunt 22 Aug 00

Deviation Reviewed and Registered by QAU/Date: Entabesin 10/9/00

Study Director (if applicable)

MEDICAL RESEARCH AND EVALUATION FACILITY

DEVIATION FORM

Study and/or Facility record affected

Study Title: Protection of Guineas Pigs by Passive Immunization with Human Botulinum Immune Globulin Obtained Following Vaccination

Study Number: G155553A

Record: _____ Deviation No. (Assigned by QAU): DR-264

Type of Deviation

☐ GLP (Section): _____

☐ SOP (Number): _____

☒ Protocol (Number): MREF Protocol 130

☐ Method (Number): _____

☐ Other: _____

Date of Deviation(s): 15 July 98 – October 2000

Description of Deviation: Reference Protocol 130, page 24, 4th item (††).

Serum samples received under Task 161 have never been assayed in duplicate. Samples from volunteers were not analyzed in duplicate on different days and averaged as stated.

Samples were analyzed individually only once under Task 161 prior to processing.

Cause of Deviation: Samples assayed under Task 161 were not analyzed in duplicate.

Impact of Deviation: None. The plasma samples were subsequently commingled into larger batches for purification and lyophilization. The final product (purified and lyophilized plasma) was assayed for neutralizing antibody by lot. Assay results prior to co-mingling were only used as a screening mechanism to verify the immunogenic responses of the recipients.

Corrective Action: None

NA

Rev
20 Dec 00

CA No. Assigned by QAU (if applicable):

If deviation is planned, effective date:

Deviation form Prepared by/Date: Robert E. Hunt / 20 Dec 00

Deviation Reviewed and Corrective Action Accepted by/Date: Robert E. Hunt / 20 Dec 00

Deviation Reviewed and Registered by QAU/Date: ENB/USN/12/29/00

Study Director (if applicable)

APPENDIX C

Battelle SOP MREF. X-014

**Standard Operating Procedure (SOP) for the Detection and Quantitation of
Botulinum Toxins and Antitoxins (Antibodies)**

BATTELLE COPY

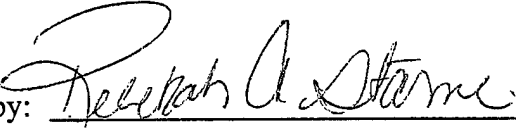
JUL 30 1999

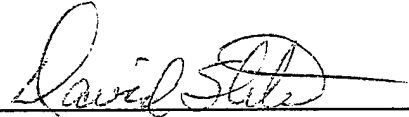
Manual Number: 464

Battelle SOP Number: MREF. X-014-06

Page 1 of 10

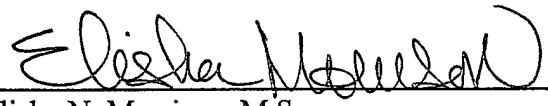
**STANDARD OPERATING PROCEDURE (SOP) FOR THE DETECTION AND
QUANTITATION OF BOTULINUM TOXINS AND ANTITOXINS (ANTIBODIES)**

Originated by:  Date 7/9/99
Rebekah A. Starner, B.S.
Researcher

Reviewed by:  Date 7-9-99
David L. Stitcher, CIH
MREF Environmental Safety and Health Officer

Approved by:  Date 7-9-99
James E. Estep, D.V.M., Ph.D.
Manager
Medical Research and Evaluation Facility

Reviewed and Registered by QAU:

 Date 7/28/99
Elisha N. Morrison, M.S.
Senior Quality Assurance Specialist

Battelle's Medical Research and
Evaluation Facility (MREF)
505 King Avenue, JM-3
Columbus, Ohio 43201-2693

BATTELLE COPY

Manual Number: 46

Battelle SOP Number: MREF. X-014-06
Page 2 of 10

I. Scope

This SOP applies to all personnel who perform the botulinum mouse neutralization and potency assays. This includes study preparation, toxin/antibody preparation, animal weighing, dosing, and observations.

II. Purpose

The mouse bioassay determines botulinum potency and the amount of toxin-specific neutralizing antibodies to a specific botulinum toxin serotype.

III. References

- A. FSP SOP BIO-005, Safe Handling and Storage of Etiological Agents.
- B. Battelle SOP Number: MREF. X-013, Intramuscular, Intraperitoneal, and Subcutaneous Injections of Etiological Agents and Vaccines into Vertebrate Laboratory Animals.
- C. Material Safety Data Sheets (MSDS) for Botulinum toxins.
- D. Kautter, D. A., Solomon, H.M., and Rhosehamel, E.J., (1992) FDA Bacteriological Manual. 7th Ed.
- E. Ryan, T.P. (1989), Statistical Methods for Quality Improvement. John Wiley and Sons, New York.
- F. SAS (1995), SAS/QC Software: Usage and Reference. SAS Institute Inc., Cary, N.C.
- G. Method Number 11/Microbiology, Method for the preparation and storage of botulinum toxins.
- H. Method Number 13/Microbiology, Method for the detection and quantitation of proteins using the BCA protein assay.

IV. Definitions

- A. LD₅₀ - the quantity of toxin that causes the death of half the animals dosed within a given time frame.
- B. MIPLD₅₀ Unit - the quantity of toxin which when injected intraperitoneally into mice causes the death of half the animals within ninety-six hours. One unit of toxin is the LD₅₀. Botulinum toxins are quantitated by their potency or activity, not weight.
- C. Millipore water - water purified using the Milli-RO Plus® polished with the Milli-Q PF Plus® system (Millipore, Bedford, MA).
- D. TPCK - L-tosylamide-2-phenylethyl chloromethyl ketone - agent used to reduce the chymotrypsin contamination of trypsin.
- E. ED₅₀ -- the amount of antitoxin/antibodies that protects half of the animals dosed from a predetermined toxin challenge dose.

V. Procedures

- A. Needed Materials. Botulinum toxins, antitoxins, antitoxin standards [World Health Organization (WHO) Antitoxin Standards. PerImmune, Inc. (PI) Antitoxin Standards], syringes, needles, mice, sterile gel phosphate buffer, test tubes, pipets and tips, vortex, centrifuge, centrifugal concentrators, pH meter, conductivity meter, trypsin, dibasic sodium phosphate, sodium chloride, hydrochloric acid (HCl), filter sterilizing flasks, vacuum pump, bovine serum albumin (BSA), and gloves.
- B. Hazard Information. Botulinum toxins attack the presynaptic terminal of the peripheral nerves, thereby blocking the release of acetylcholine and preventing muscle contractions. The botulinum toxins are among the most potent of toxins. An estimated lethal dose for humans is about 1 µg/kg by ingestion and 1 ng/kg by injection (assuming humans are at least as sensitive as mice). For mice, aerosol doses of botulinum toxins are typically 20-80 fold less toxic than injections. It is the responsibility of the users to read and understand the MSDS before handling botulinum toxins.
- C. Maintain all documentation in the appropriate study file.
- D. Preparation of Solutions (all volumes and amounts may be proportionately increased or decreased, as needed).

1. Sterile gel phosphate buffer - dissolve 2 g of gelatin and 4 g of dibasic sodium phosphate in 900 mL of millipore water. Heat the solution until the gelatin dissolves and then cool to room temperature. Adjust the pH to approximately 6.2 with HCl and bring the volume to 1 L. Aliquot into smaller volumes and autoclave for ~30 min. Cool and store refrigerated for up to 6 months. Document preparations (i.e., Form Number MREF Microbio.frm-100).
 2. Trypsin stock solution (10 mg/mL) - dissolve 10 mg of trypsin (TPCK-treated) in one mL of gel phosphate buffer. Prepare the solution just prior to use. Document preparation (i.e., Form Number MREF Microbio.frm-095).
 3. Phosphate buffered saline (PBS), 50 mM sodium phosphate, 0.2 M NaCl - dissolve 5.7 g of sodium phosphate monobasic (NaH_2PO_4), 1.3 g of sodium phosphate dibasic (Na_2HPO_4) and 11.0 g of NaCl in approximately 900 mL of millipore water. Check the pH of the solution. If the pH is outside the range of 5.9 - 6.3, discard and remake it. Adjust the pH to approximately 6.2 with NaOH or HCl. Bring the volume to one liter. Aliquot into the desired volumes and autoclave for at least 20 minutes. Store the solution refrigerated for up to six months. Document preparation (i.e., Form Number MREF Microbio.frm-067).
 4. Bovine serum albumin (BSA) solution (50 mg/mL) - dissolve 5.0 g of BSA in 100 mL of millipore water and filter sterilize with a 0.22 μm filter. Store the solution at less than -20 degrees C for up to 6 months. Thaw just prior to use. Document preparation on Form Number MREF Microbio.frm-097.
- E. Preparation of Toxin [**WARNING:** Botulinum toxins are extremely dangerous. Perform all manipulations in Class II or III Biological Safety Cabinet (BSC), unless specifically stated otherwise. Wear gloves at all times and take extreme care when using a needle and syringe for injecting animals]. See reference III. G. for specific toxin preparation procedures.
1. The botulinum toxins are received as ammonium sulfate precipitates in one batch for each individual serotype. Centrifuge the solutions to a pellet in a fixed angle rotor for approximately 15 min. Remove and discard the supernatant (treat as contaminated waste). The supernatant may be tested for potency using the LD₅₀ assay, if desired.
 2. Dissolve the pellet in sterile PBS. Remove the excess ammonium sulfate by centrifugal concentration and reconstitution until the conductivity of the sample diluted to 1/100 with millipore water is within ~20 percent of the conductivity of the reconstitution buffer diluted 1/100 with millipore water, or until completing

seven concentrating spins.

3. Record the toxin preparation and aliquotting (i.e., Form Number MREF Microbio.frm -113).

F. Determination of MIPLD₅₀ Units (potency)

1. Following the dilution sheet (i.e., Form Number MREF Microbio.frm-100), set up multiple graded dilutions (using sterile gel phosphate buffer) of the botulinum toxin with the middle concentration containing an estimated 1 MIPLD₅₀ unit per inoculum. Determine the dilution factor based on the need of the particular experiment. For example, the initial potency determination is performed using a broad spectrum dilution series (e.g., 4-fold), in order to determine a more narrow range, while particular experiments may require a more compacted series (e.g., determination of aerosolized toxin) for a more accurate potency determination. It may first be necessary to perform a preliminary experiment in order to narrow the dilution range.
2. Inject mice intraperitoneally (See reference III.B., above) with approximately 0.5 mL of toxin at each concentration. The number of mice injected per group is based on the required precision. Refer to validation results (MREF Task 39) to determine the level of precision that is attained with differing numbers of mice.
3. Record the number of dead mice as well as live mice per group during daily clinical observations for a period of four days and at 96 ± 2 hr, or as specified in the study protocol. For each observation, if the total number of mice is different than the previous observation period, document this on the mouse observation form (i.e., Form Number MREF BL3.frm-021).
4. Determine the LD₅₀ dose based on the number of mouse deaths after 96 ± 2 hours. Determine the MIPLD₅₀ unit per mL in using probit analysis.
5. Depending on the strain of *C. botulinum*, some toxins such as botulinum E and sometimes C and D are excreted from the bacterial cell as un-nicked (inactive) toxins and require proteolytic treatment with trypsin prior to their use in bioassays. For toxins isolated from these strains of *C. botulinum*, use the following procedures.
 - a. Determine the protein concentration of the botulinum toxin using colorimetric or spectrophotometric procedures (See reference III.H.).

- b. Adjust the total protein content of the toxin to approximately 0.5 mg/mL (e.g., a 1 mL toxin sample with a protein concentration of 100 μ g/mL would require an addition of 400 μ g of protein or 8 μ L of the BSA solution).
- c. To the protein adjusted toxin sample, add 10 μ L of the trypsin solution (add 50 μ L of the trypsin to a 5 mL toxin sample, etc.).
- d. Incubate the toxin/trypsin/BSA solution at approximately 37 degrees C for approximately one hour.
- e. Once incubation is complete, dilute the sample for the assay or place on ice until ready to use.
- f. Determine the MIPLD₅₀ units. Use treated toxins for all subsequent assays and experiments.
- g. Record steps b through e on the dilution sheet (i.e., Form Number MREF Microbio.frm-100).

G. Determination of the Antibody (antitoxin) Concentrations

1. Prepare a solution in gel phosphate buffer of standard botulinum toxin, so that one mL aliquots contain the predetermined toxin challenge dose. Toxin concentrations are expressed as a dilution factor of the 2A toxin preparations. Maintain the current dilution factors for each serotype on Form Number MREF Micro.bio-118.
2. Prepare a series of graded dilutions of the antibodies or antitoxins (see Form Number MREF Micro.bio-118 for the midpoint for the current standards of use) in one mL volumes. Add one mL of the toxin challenge dose and mix, taking care not to generate foam [if more than 2 mL total volume is needed to dose the mice, a larger volume of antitoxin/antibodies is prepared and challenged with an equal volume of toxin challenge dose (i.e., 1.5 mL of antitoxin/antibody is combined with 1.5 mL of toxin challenge dose)]. Document this on the dilution sheets (i. e., Form Number MREF Microbio.frm-100).
3. Allow the antigen/antibody mixtures to stand for 60-120 min at room temperature.
4. Inject a dose of the mixture (~0.2 mL) intraperitoneally into mice. The number of mice dosed per group is based on the required precision. Refer to validation

BATTELLE COPY

results (MREF Task 39) to determine the level of precision attained with differing numbers of mice.

5. Record the number of dead mice as well as live mice per group during daily clinical observations as described in paragraph V.F.3.
6. The end point of the titration is based on the death or survival of the mice after 96 ± 2 hr. Calculate the results using probit analysis.

H. Quality Control

1. All Assays

- a. Weigh the mice and assign them to a study. On the day of the study, mice must weigh 18.0-22.0 g for MIPLD₅₀ assays and 17.0-23.0 g for neutralization assays.
- b. Once a group of study mice is assembled, mice are chosen in an arbitrary fashion, assigned to a treatment group, injected, and placed in their home cage.
- c. During an experiment, open only one cage at a time to ensure animal mixing does not occur between cages.
- d. After injection and before returning to the appropriate cage, visibly mark all animals to ensure that each animal is injected only once. If deemed necessary by the study protocol, mark the injection order of the animal.
- e. Once treated mice are placed into their home cage, do not remove them for any reason with the following exceptions: (1) Remove dead mice during daily observations; (2) In the rare event that a mouse needs to be removed and treated for non-botulinum related reasons, properly mark and identify it upon removal to ensure its return to the proper cage.
- f. If animals are randomized before use, take proper steps to ensure the identification of individual animals throughout the study, unless otherwise specified in the study protocol.
- g. Do not return escaped, unmarked animals to study.

- h. For the experiment to be valid, not more than 15 percent of the test mice are removed from the study for non botulinum reasons (e.g., escaped mice, injured mice, etc.) during the experiment.
- i. The Study Director will determine whether or not to repeat an assay that fails to meet the acceptance criteria.

2. MIPLD₅₀ Assays

- a. Perform a buffer-only (BC) negative control with each set of experiments to show that the buffer has no ill effects on the mice.
- b. For toxin treated with trypsin, perform a buffer/trypsin/ BSA control (TC) to show this mixture has no ill effects on the mice.
- c. For experiments with toxins treated with trypsin, perform/test an untreated control (NTC) at approximately ten times the highest concentration of the trypsin treated toxin.
- d. Use only mice weighing between 18.0-22.0 g for LD₅₀ assays.
- e. For each set of experiments, as a positive control to show that the toxin is potent, one group of mice receives doses containing approximately 20 MIPLD₅₀ units of toxin, or alternatively, a standard curve of serially diluted stock solutions is performed.
- f. At a minimum, one experimental group must have 50 percent or greater survival and one group, 50 percent or greater mortality for the experimental results to be accepted (i.e., the experimental curve must touch or span the 50 percent mortality level).

3. Antibody detection

- a. For each set of experiments, perform a standard curve using either serially diluted WHO or PI antitoxin standards, or alternatively, standards calibrated with the WHO or PI antitoxins.
- b. For the test to be valid, the observed ED₅₀ of the standard curve cannot vary more than 2 dilution units from the expected ED₅₀. For test substances, the observed ED₅₀ for the test curve must be within the range of doses tested.

BATTELLE COPY

Manual Number: 464

Battelle SOP Number: MREF. X-014-06

Page 9 of 10

- c. For the test curve to be acceptable, there must be less than 50 percent lethality at the highest concentration of test serum (or antitoxin, in the case of the standard curve) and more than 50 percent lethality at the lowest concentration.
- d. Perform a toxin only control (TOC) at the same concentration as the challenge dose for each set of experiments in order to show that the toxin is potent. Perform a BC negative control for each set of experiments.
- e. Use only mice weighing between 17.0-23.0 g for neutralization assays.
- f. As the toxins may slowly degrade over time, the dilution factors for the toxin challenge doses can be periodically reoptimized by titration against a fixed antitoxin amount (standard curve midpoint: see Form Number MREF Micro.bio-118). If ED_{50} values begin to show a clear pattern of deviation from the expected value (see paragraph V.H.3.h. below), calculate new dilution factors by constructing titration curves in at least triplicate (on three separate days) and averaging the results to determine the new dilution factors. Once established, modify Form Number MREF Micro.bio-118 to reflect the new values. In the interim period, place a memo in any affected studies detailing the altered dilution factors.
- g. Toxin challenge doses are reoptimized (see V.H.3.f) at a minimum every six months ($\pm$ one month) for all serotypes. This period may be extended if the assay is not in use for a given serotype; however, reoptimization must occur before using the assay to generate study data. Included in these experiments is a determination of toxin potency. If the challenge dose (in $MIPLD_{50}$) decreases substantially from historical levels, the antibody standards are evaluated for loss of activity.
- h. A statistical process control method, CUSUM (See references III.E. and F.), is used to monitor assay variability. Parameters for the CUSUM method are determined and noted in a memo initiated by the statistical staff. An assessment of the assay procedures will be initiated when the CUSUM method indicates the assay may be out of control. If the CUSUM method indicates reoptimization is necessary or if three out of six curves do not meet specifications, as outlined in paragraphs V.H.3.b. and c., the assay is not used until reoptimization of the challenge dose has been completed.
- i. An estimate of assay variability is required for the CUSUM method. Historical data are used to estimate assay variability about the target midpoint for the standard curve. Use all available data for this estimate, except points

where the assay is suspected to be out of control or points that are statistical outliers (determined by residual analysis). Variability estimates and CUSUM parameters may be adjusted when the assay is reoptimized periodically, or as needed, and changes noted in a memo initiated by the statistical staff.

I. Hazard Analysis

1. An accidental needle stick while injecting a mouse is a route of toxin exposure for workers.
2. Simple manipulations (e.g., opening the lid of a closed vessel, pipetting the solutions) cause aerosolization of toxins in solutions and could produce a toxin exposure.

J. Safety Precautions

1. Anyone working with the botulinum toxins or at risk of toxin exposure must be vaccinated with the botulinum toxoid available from the Centers for Disease Control and Prevention (CDC) and produce a titer to botulinum toxin A. Vaccinated individuals have an estimated 250 times the protection of nonimmunized individuals for Bot A (e.g., they are protected against 250 human LD₅₀ dose of botulinum toxin).
2. Perform toxin manipulations in either a Class II or III BSC, if working with concentrations >5000 MIPLD₅₀ units/mL (approximately one nonimmunized human LD₅₀ based on weight). Procedures performed outside a BSC (e.g., intraperitoneal injections of toxin for LD₅₀ assays or neutralization assays as per reference III.B. above), must follow an approved Battelle SOP. Perform centrifugation only in sealed rotors. Once centrifugation is complete, remove the rotor from the centrifuge and open it in a Class II or III BSC.
3. Only trained personnel inject mice intraperitoneally with toxin for potency or antibody detection. Use extreme care since additional personal protective equipment (besides gloves) would reduce dexterity and may increase the risk of a needle stick. The maximum dose by accidental needle stick is estimated to be 100 MIPLD₅₀ of toxin, or 0.03 human LD₅₀ units (adjusting for the average weight of a human and assuming maximum injection volume of 0.1 mL), which is less than the protection level conferred by immunization.

APPENDIX D

Statistical Reports for Mortality Data



Project Number G1555-53ASTAT (3104)

Internal Distribution

Lee/Dept. Files
NA Niemuth
MC Matthews
JR Holdcraft
BK Pierce*
RMO

* (memo only)

Date December 1, 2000

To **Bob Hunt**

From Claire Matthews^(CM)/Nancy Niemuth

Subject **Statistical Analysis for MREF Task 97-53
(Final Report)**

(S:\niem\BL3\Task53\97-53 Final Report.doc)

The attached report provides the results of statistical analysis of QA-reviewed guinea pig lethality data collected under MREF Task 97-53. This report replaces our previous version dated August 14, 2000, which contained results of analyses performed on preliminary data.

Please call me at 424-5595 if you have any questions on the results provided.

MCM/NAN:lj
Attachment

For Review and Approval

	Name	Internal	Date
Originator	Claire Matthews	CM	12-1-00
Concurrence	Nancy Niemuth		
Approved	Ben Pierce	BKP	12/01/00

Sent Via: Interoffice Mail

STATISTICAL REPORT FOR MREF TASK 97-53

December 1, 2000

Overview

Experiments were conducted under MREF Task 97-53 to determine the protection provided by primary and primary-plus-booster inoculation series of Pentavalent Botulinum Toxoid vaccine against the toxic effects of botulinum serotype A using human botulinum immune globulin in a passive protection model. Guinea pigs (GP) were treated with one of five immune globulin (IG) types to achieve circulating serum antibody concentrations of approximately 0.06 U/mL for botulinum serotype A. These five IG types are abbreviated as:

- PBIGA (primary inoculation series, Lot A),
- PBIGB (primary inoculation series, Lot B),
- PBIGAB (primary inoculation series, a mixture of Lots A and B),
- BBIG (primary booster series plus inoculation, a mixture of Lots A and B), and
- CIG (control immune globulin).

Twenty-four hours after these treatments, animals were challenged with serotype A. Sentinel animals treated with the same five IG types were used to determine circulating antibody levels using the mouse neutralization assay.

Relevant data collected under Task 97-52 were included in the analysis for comparison. This historical data consisted of the dose-lethality results from guinea pigs treated with BIG or VIG (vaccinia immune globulin) and challenged with a similar dose of serotype A.

Methods

Probit dose-response models were fitted to the guinea pig lethality data as a function of the GP doses, separately for each IG preparation. Estimated parameters of the probit dose-response models were used to calculate the LD₅₀ for each IG type, together with 95 percent confidence intervals using Fieller's method (Finney, 1971). LD₅₀ ratios and protective ratios with 95 percent confidence intervals were calculated for various IG type combinations, including ratios relative to the LD₅₀s from Task 97-52. The LD₅₀ ratio is significantly different from unity if its confidence interval does not overlap the value of 1.0, in which case the LD₅₀s from the two IG types are demonstrated to be significantly different from each other. Probit dose-response models were fitted using the SAS (V8) PROBIT procedure.

Doses were expressed as MIP LD₅₀ units per animal and were calculated as:

$$\text{GP dose} = \text{injection volume (mL)} * \text{dose dilution} * \text{toxin potency},$$

where toxin potency is the MIP LD₅₀ per mL established by a mouse potency assay run on the challenge day.

Two of the four mouse potency experiments (#91004 on 3/29/00 and #91005 on 4/12/00) each had one toxin concentration group resulting in a lethality rate that was considered an outlier. Therefore, the probit analysis that computed the MIP LD₅₀ was repeated for those two experiments after removing the outlier group. The toxin potency values attached after making these adjustments were more consistent with historical values. The table in Attachment A presents a summary of these calculations for the mouse potency experiments.

The statistical analysis was repeated using "adjusted" GP doses calculated using the "adjusted" toxin potency for the two experiments. For guinea pigs matched with the other two mouse potency experiments (#91003 on 3/15/00 and #91008 on 4/26/00), the adjusted GP dose was the same as the unadjusted GP dose.

Sentinel Animals

The mouse neutralization assay was used to determine the serum antibody concentrations (SAC) achieved in the sentinel animals passively immunized with the various IG types. All but one of the BIG-treated animals achieved quantifiable SACs. The single exception was for guinea pig 10; the corresponding mouse neutralization experiment #6089 (4/27/00) tested only one undiluted serum concentration and failed to meet the acceptance criteria. This guinea pig was excluded from the statistical analysis. As expected, the SACs for CIG-treated animals could not be quantified and therefore this group was also excluded from the statistical analysis.

Descriptive statistics (means, standard deviations, and 95% confidence bounds for the means) were computed for the SACs from sentinel animals for each BIG-treated group, including the BIG group from Task 97-52. A one-way analysis of variance (ANOVA) was used to test the significance of IG type effects upon the SACs, and a Tukey's Multiple Comparison Test was used to rank and group the IG types according to their mean SACs.

Avidity

For avidity experiments, the number of MIP LD₅₀ units per injection was calculated as:

$$\text{MIP LD}_{50} \text{ units/injection} = \text{LD}_{50} * \text{toxin potency} * 0.25,$$

where the LD₅₀ is estimated from the dose-response curve fitted to the avidity experiment data, and the toxin potency is the MIP LD₅₀ per mL established by a mouse potency assay run on the same day.

Results

Tables 1a and 1b present results from probit analysis performed on guinea pig dose-lethality data using GP doses based on unadjusted and adjusted mouse potency data, respectively. The summary statistics shown are the probit slope, estimated LD₅₀, 95 percent confidence interval, and percent half-width of the confidence interval. Figures 1a and 1b display dose-lethality curves predicted by the probit analysis based on the unadjusted and adjusted GP doses respectively; the curves for the five IG types from Task 97-53 and two IG types from Task 97-52 are shown together. Tables 2a and 2b present estimated LD₅₀ ratios and protective ratios for various pairs of IG types, based on the LD₅₀ estimates shown in Tables 1a and 1b respectively.

The LD₅₀ ratios, confidence intervals, and p-values in Tables 2a and 2b demonstrate that the seven IG types fell into several statistically distinct clusters, as follows:

- The LD₅₀s were not statistically different among the PBIGA, PBIGB, PBIGAB, and BBIG groups. The geometric mean of these four LD₅₀s was approximately 115,000 MIPLD₅₀ units per animal for the analysis on unadjusted GP doses, and was approximately 84,000 MIPLD₅₀ units per animal for the analysis on adjusted GP doses.
- The LD₅₀s from the PBIG- and BBIG-treated animals in Task 97-53 were significantly greater than the LD₅₀ from the BIG-treated animals in Task 97-52.
- The LD₅₀s from the PBIG-, BBIG-, and BIG-treated animals in both Tasks 97-53 and 97-52 were significantly greater than those from both control groups (CIG and Task 52 VIG).
- The LD₅₀ from the Task 97-53 CIG control group was significantly greater than that from the Task 97-52 VIG control group.

The same patterns of group similarities and differences can be seen in Figures 1a and 1b. The LD₅₀s for the seven IG type groups correspond to the GP doses where each curve crosses the horizontal reference line at 0.5 proportion lethality.

Sentinel Animals

Table 3 presents descriptive statistics for SACs in five groups of BIG-treated animals from Task 97-52 and Task 97-53, footnoted with the results from the Tukey comparisons. Figure 2 displays SAC values for individual animals (dots) and mean SACs (dashes) for the same five groups. The horizontal reference line is shown at the target SAC of 0.06 U/mL.

The ANOVA performed on the SACs observed in sentinel animals demonstrated that significant differences existed among the five groups ($p < 0.005$). The Tukey's Multiple Comparison Test showed that mean SACs were not statistically different among the primary series injection groups (PBIGA, PBIGB, and PBIGAB). The average of these three group means

was approximately 0.057 U/mL. The mean SAC of 0.082 U/mL in the booster injection group was significantly greater than the mean SACs in the three primary injection groups. By comparison, the mean SAC level in the Task 97-52 BIG animals was intermediate in value (approximately 0.073 U/mL) and was not significantly different from any of the Task 97-53 groups.

Avidity

Figure 3 presents the neutralizing potency at the ED₅₀ relative to the L+/10 dilution using PBIGA, PBIGB, BBIG, PBIGAB, and BIG for Serotype A.

Conclusions

For the potency experiments, the same patterns of similarities and differences among IG types were evident regardless of whether the analysis was performed on the unadjusted or adjusted GP doses. Similar levels of protection were provided by vaccine Lots A and B for primary series injections. The primary series and primary series-plus-booster injection also afforded similar levels of protection. For both sets of analyses, the geometric mean LD₅₀ taken across the PBIGA, PBIGB, PBIGAB, and BBIG groups was more than 10,000 times greater than the corresponding LD₅₀ for the corresponding CIG control group. By comparison, the protection provided by the BIG treatment in Task 97-52 was significantly lower than for any of the IG treatments in Task 97-53.

The analysis of the SACs observed in sentinel animals demonstrated that the average SAC achieved in the booster injection group was significantly greater than those in the three primary injection groups (PBIGA, PBIGB, and PBIGAB). The average SACs achieved in the three primary injection groups were statistically similar to each other. The average SAC for BIG-treated animals in Task 97-52 was intermediate in value and not significantly different from any of the Task 97-53 groups.

References

Finney, D.J. (1971). Probit Analysis, Third Edition, Cambridge University Press, Cambridge, England.

Table 1a. Estimated LD₅₀s and Fieller's 95 Percent Confidence Intervals for Each IG Type Resulting from Analysis Performed on Unadjusted GP Doses.

IG Type Used with Serotype A	N	Probit Slope (*)	LD ₅₀ (MIP LD ₅₀ Units per animal)	Fieller's 95 Percent Confidence Interval for LD ₅₀	Percent Halfwidth (%)
PBIGA	40	1.7	83,672	(6328, 233183)	136
PBIGB	30	5.7	149,984	(102657, 240094)	46
PBIGAB	40	1.8	102,576	(23972, 205858)	89
BBIG	30	5.6	134,540	(91029, 209206)	44
CIG	40	4.1	11.39	(8.48, 19.86)	50
Task 97-52, BIG	40	1.4	1,902	(460, 3844)	89
Task 97-52, VIG	40	2.6	3.98	(1.72, 6.00)	54

(*) All probit slopes were significantly greater than zero ($p \leq 0.05$).

Table 1b. Estimated LD₅₀s and Fieller's 95 Percent Confidence Intervals for Each IG Type Resulting from Analysis Performed on Adjusted GP Doses.

IG Type Used with Serotype A	N	Probit Slope (*)	LD ₅₀ (MIP LD ₅₀ Units per animal)	Fieller's 95 Percent Confidence Interval for LD ₅₀	Percent Halfwidth (%)
PBIGA	40	3.3	67,295	(34821, 97290)	46
PBIGB	30	5.8	96,693	(68372, 151559)	43
PBIGAB	40	3.0	86,447	(41347, 126897)	49
BBIG	30	5.7	86,894	(61059, 133419)	42
CIG	40	7.8	8.15	(6.84, 12.1)	32
Task 97-52, BIG	40	1.4	1,902	(460, 3844)	89
Task 97-52, VIG	40	2.6	3.98	(1.72, 6.00)	54

(*) All probit slopes were significantly greater than zero ($p \leq 0.05$).

Table 2a. Estimated LD₅₀ Ratios And Protective Ratios Resulting from Analysis Performed on Unadjusted GP Dose Data.

Description of Comparison	LD ₅₀ s Compared (Numerator IG Type/ Denominator IG Type)	LD ₅₀ Ratio (95% Confidence Interval)	P-value for Comparing Ratio to 1.0
Compare IG from primary series to primary series-plus- booster injections	PBIGA / BBIG	0.622 (0.331, 1.17)	0.14
	PBIGB / BBIG	1.11 (0.771, 1.61)	0.56
	PBIGAB / BBIG	0.762 (0.397, 1.46)	0.41
Compare IG from different vaccine lots for primary series injections	PBIGB / PBIGA	1.79 (0.955, 3.36)	0.069
	PBIGAB / PBIGA	1.23 (0.536, 2.81)	0.63
	PBIGB / PBIGAB	1.46 (0.762, 2.81)	0.25
Compare IG from primary series injections to control IG	PBIGA / CIG	7350 (3810, 14200)	< 0.0001
	PBIGB / CIG	13200 (8710, 19900)	< 0.0001
	PBIGAB / CIG	9010 (4570, 17800)	< 0.0001
Compare IG from primary series-plus-booster injections to control IG	BBIG / CIG	11800 (7810, 17900)	< 0.0001
Compare IG controls between Task 52 and Task 53	Task 52 VIG / CIG	0.349 (0.197, 0.617)	0.0003
Compare Task 52 BIG to Task 53 IG controls	Task 52 BIG / CIG	167 (72.2, 387)	< 0.0001
Compare IG from primary series and primary series- plus-booster injections to BIG from Task 52	PBIGA / Task 52 BIG	44.0 (16.8, 115)	< 0.0001
	PBIGB / Task 52 BIG	78.8 (34.8, 179)	< 0.0001
	PBIGAB / Task 52 BIG	53.9 (20.3, 143)	< 0.0001
	BBIG / Task 52 BIG	70.7 (31.2, 160)	< 0.0001

Table 2b. Estimated LD₅₀s Ratios And Protective Ratios Resulting from Probit Analysis Performed on Adjusted GP Dose Data

Description of Comparison	LD ₅₀ s Compared (Numerator IG Type / Denominator IG Type)	LD ₅₀ Ratio (95% Confidence Interval)	P-value for Comparing Ratio to 1.0
Compare IG from primary series to primary series-plus- booster injections	PBIGA / BBIG	0.774 (0.512, 1.17)	0.23
	PBIGB / BBIG	1.11 (0.777, 1.59)	0.56
	PBIGAB / BBIG	0.995 (0.628, 1.58)	0.98
Compare IG from different vaccine lots for primary series injections	PBIGB / PBIGA	1.44 (0.950, 2.17)	0.086
	PBIGAB / PBIGA	1.28 (0.776, 2.13)	0.33
	PBIGB / PBIGAB	1.12 (0.705, 1.77)	0.63
Compare IG from primary series injections to control IG	PBIGA / CIG	8260 (5660, 12100)	< 0.0001
	PBIGB / CIG	11900 (8640, 16300)	< 0.0001
	PBIGAB / CIG	10600 (6910, 16300)	< 0.0001
Compare IG from primary series-plus-booster injections to control IG	BBIG / CIG	10700 (7770, 14600)	< 0.0001
Compare IG controls between Task 52 and Task 53	Task 52 VIG / CIG	0.488 (0.294, 0.810)	0.0056
Compare Task 52 BIG to Task 53 IG controls	Task 52 BIG / CIG	233 (105, 519)	< 0.0001
Compare IG from primary series and primary series-plus- booster injections to BIG from Task 52	PBIGA / Task 52 BIG	35.4 (15.3, 82.0)	< 0.0001
	PBIGB / Task 52 BIG	50.8 (22.5, 115)	< 0.0001
	PBIGAB / Task 52 BIG	45.4 (19.1, 108)	< 0.0001
	BBIG / Task 52 BIG	45.7 (20.2, 103)	< 0.0001

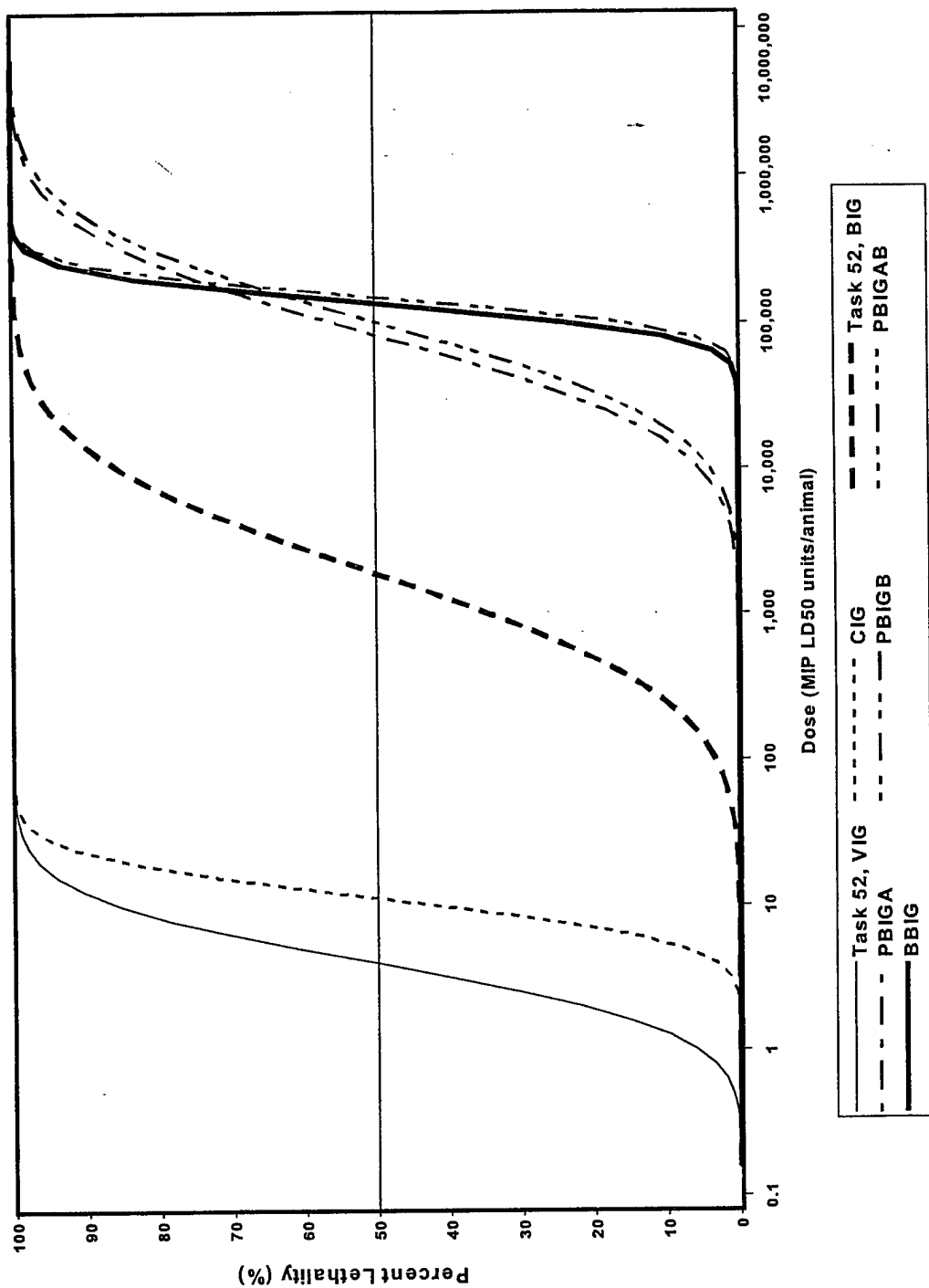


Figure 1a. Predicted Percent Lethality Plotted Against Serotype A Unadjusted Dose for Five IG Types. For Comparison, the Two Serotype A Groups from Task 97-52 are also Plotted.

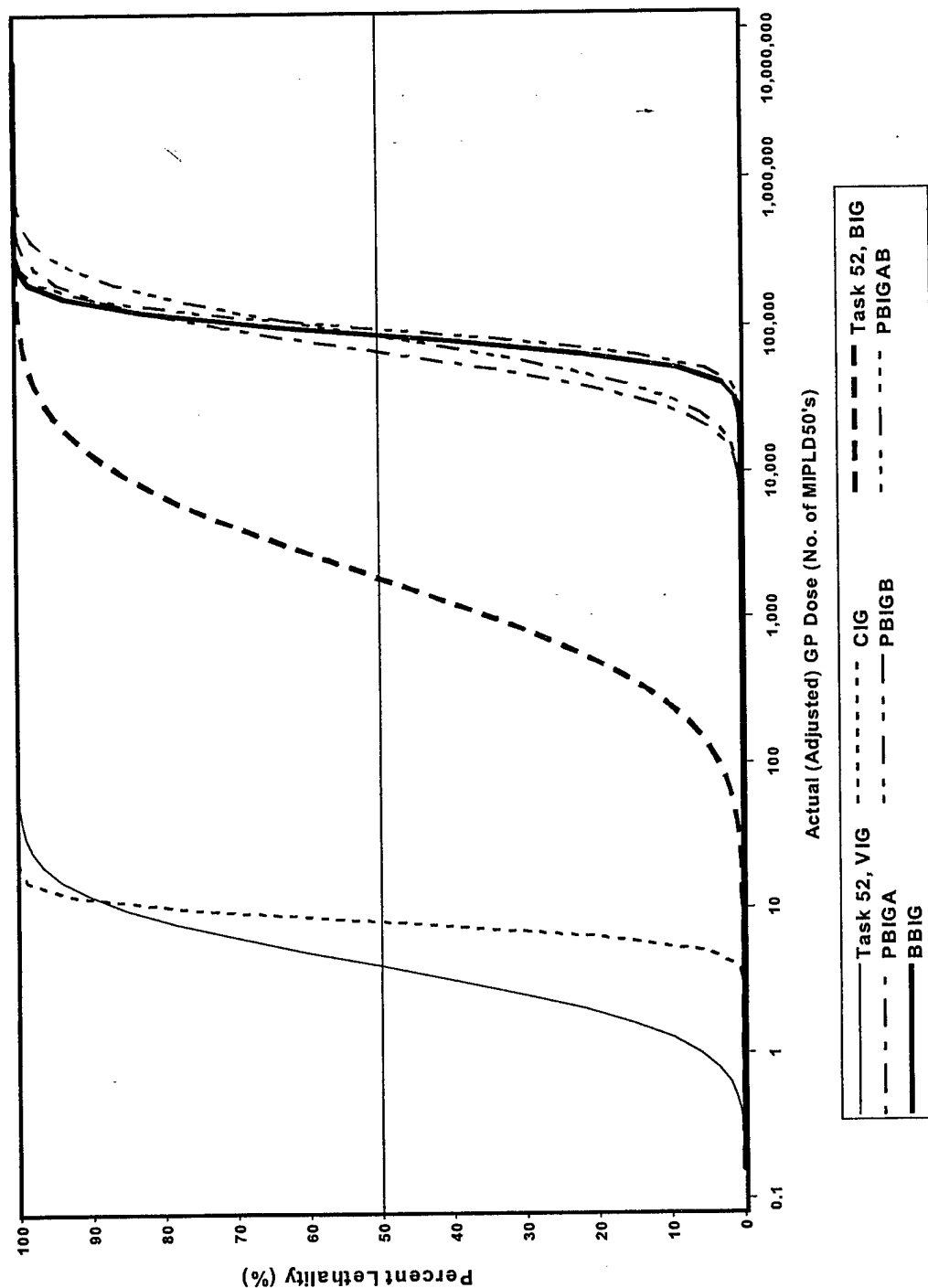


Figure 1b. Predicted Percent Lethality Plotted Against Serotype A Adjusted Dose for Five IG Types. For Comparison, the Two Serotype A Groups from Task 97-52 are also Plotted.

Table 3. Descriptive Statistics for SACs from Sentinel Animals for Each IG Type, Showing Results of Tukey Multiple Comparison Test on Mean SAC Values.

IG Type (in Increasing Order of Mean SAC)	No. of Experiments	SAC, Mean (U/mL) (*)	Standard Deviation	95% Lower Confidence Bound for Mean	95% Upper Confidence Bound for Mean
PBIGAB	16	0.0567 ^x	0.0140	0.0492	0.0642
PBIGB	11	0.0568 ^x	0.0177	0.0448	0.0687
PBIGA	15	0.0577 ^x	0.0182	0.0476	0.0678
Task 52, BIG	16	0.0726 ^{x,y}	0.0228	0.0605	0.0848
BBIG	12	0.0821 ^y	0.0187	0.0702	0.0940

(*) ^{x,y} = Letters indicating groupings of similar IG types according to Tukey comparisons. Any means not followed by the same letter are significantly different at the p=0.05 level.

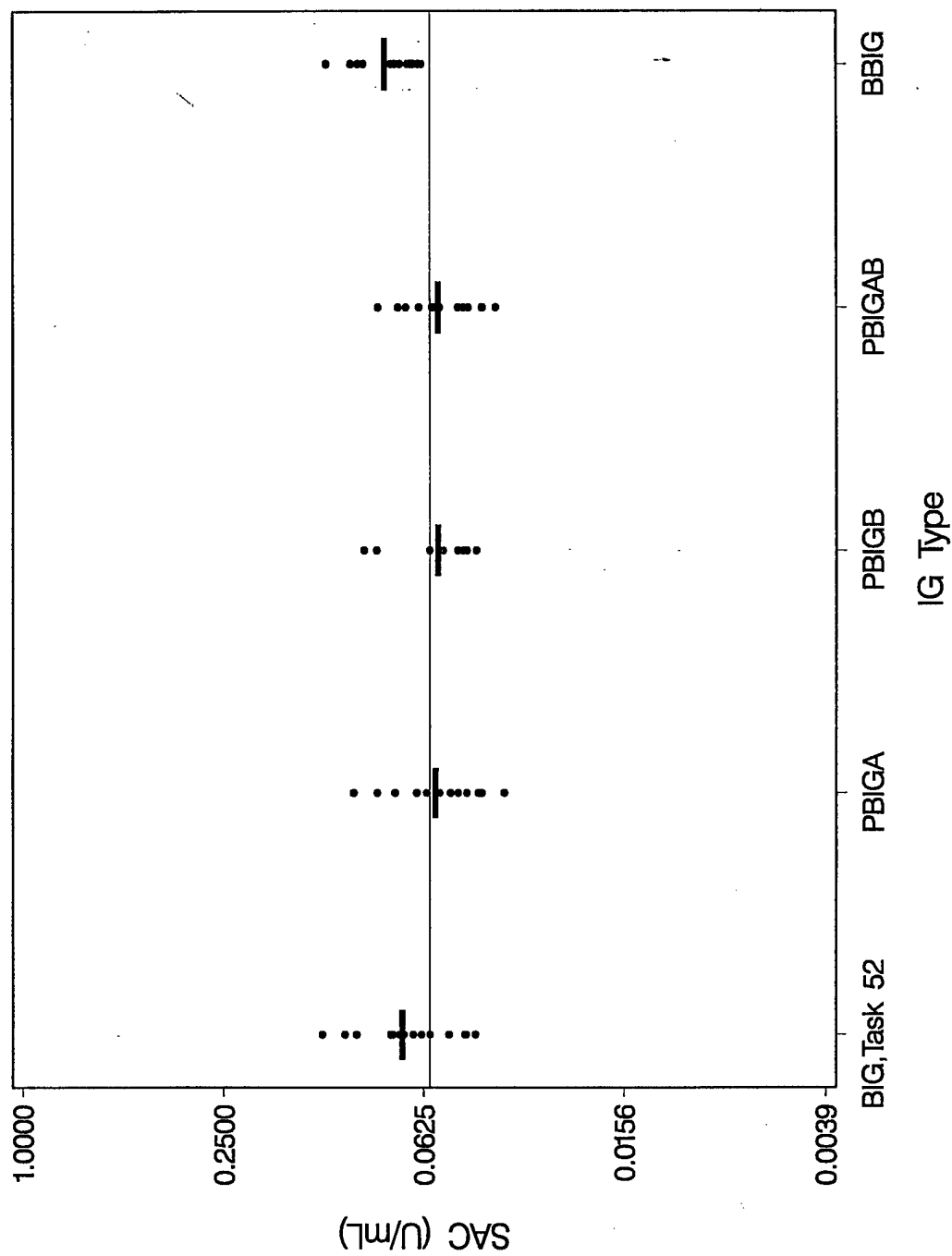


Figure 2. SACs for Sentinel Animals for Each IG Type. Dots Indicate SACs for Individual Animals; Bars Indicate Mean SACs for Each IG Type.

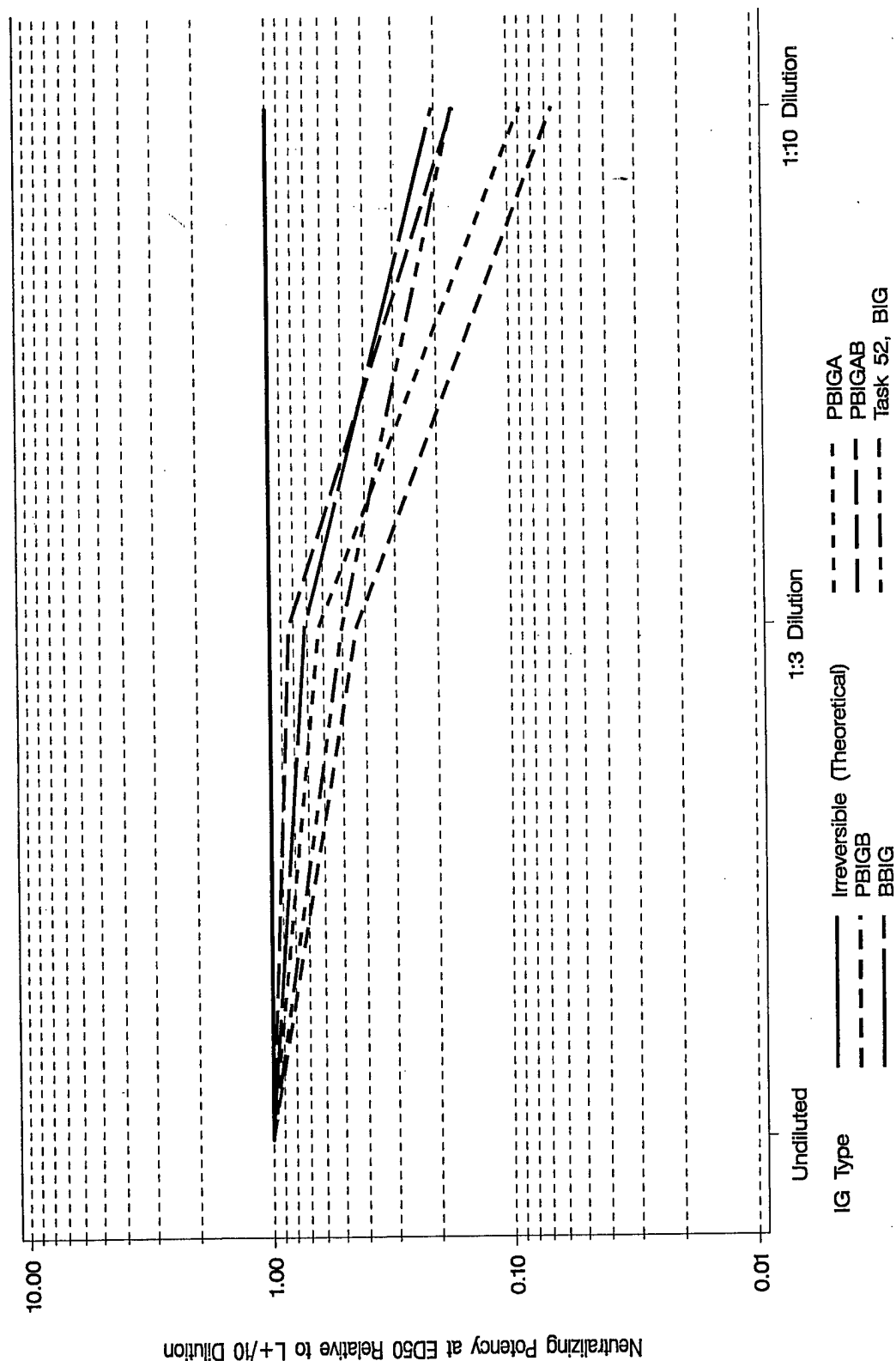


Figure 3. Neutralizing Potency at ED₅₀ Relative to L+/10 Dilution using PBIGA, PBIGB, BBIG, PBIGAB, and BIG for Serotype A.

Study No. G1555-53A

ATTACHMENT A:

**RAW DATA LISTING OF MOUSE POTENCY EXPERIMENT DATA
TOGETHER WITH ESTIMATED MIP LD50s AND TOXIN DOSES
RESULTING FROM PROBIT ANALYSIS OF UNADJUSTED AND
ADJUSTED DATA**

**Attachment A: Listing of Mouse Potency Data and Estimated MIP LD₅₀s and Toxin Doses
Resulting from Probit Analysis on Unadjusted and Adjusted Data**

Date	Expm't. No.	Group ID	Serotype A Toxin Dose Dilution	No. Dead	Estimates from Analysis on Unadjusted Data		Estimates from Analysis on Adjusted Data	
					MIP LD ₅₀ (Dose Dilution)	Toxin Potency (MIP LD ₅₀ /mL)	MIP LD ₅₀ (Dose Dilution)	Toxin Potency (MIP LD ₅₀ /mL)
3/15/00	91003	B	0.000000081	2/5	0.000000120	16,599,572	(Same as results for unadjusted data - no groups excluded from analysis)	
		C	0.000000011	0/5				
		D	0.000000016	4/5				
		E	0.000000022	5/5				
		F	0.000000031	5/5				
		G	0.000000043	5/5				
		H	0.000000061	4/5				
3/29/00	91004	B	0.000000081	0/5	0.000000134	14,976,501	0.000000207	9,645,298
		C (*)	0.000000011	5/5				
		D	0.000000016	1/5				
		E	0.000000022	4/5				
		F	0.000000031	5/5				
		G	0.000000043	3/5				
		H	0.000000061	5/5				
4/12/00	91005	B (*)	0.000000081	4/5	0.000000093	21,453,933	0.000000145	13,786,265 (same)
		C	0.000000011	0/5				
		D	0.000000016	5/5				
		E	0.000000022	4/5				
		F	0.000000031	5/5				
		G	0.000000043	5/5				
		H	0.000000061	5/5				
4/26/00	91008	B	0.000000081	0/5	0.000000228	8,767,908	(Same as results for unadjusted data - no groups excluded from analysis)	
		C	0.000000011	0/5				
		D	0.000000016	0/5				
		E	0.000000022	1/5				
		F	0.000000031	5/5				
		G	0.000000043	5/5				
		H	0.000000061	5/5				

(*) Data adjustment was made by excluding this group prior to performing probit analysis.

APPENDIX E
Statistical Reports for Clinical Data

Internal Distribution

Dept. Files
BK Pierce*
C Matthews
NA Niemuth
JR Holdcraft
RMO
* (Memo only)

Date December 5, 2000

To **Bob Hunt**

From Claire Matthews *CM*

Subject **Statistical Analysis of Clinical Signs Data
Collected in MREF Task 97-53 (Final
Report)**

S:\niem\BL3\Task53\97-53 ClinicalSigns FINAL Report.doc

The attached report summarizes the statistical analysis QA-reviewed of clinical signs data collected in MREF Task 97-53 experiments. This report replaces our previous version dated August 18, 2000, which contained results of analyses performed on preliminary data.

Please call me at 424-5595 if you have any questions on the results provided.

CM:llj

For Review and Approval

	Name	Internal	Date
Originator	Claire Matthews	<i>CM</i>	<i>12-5-00</i>
Concurrence	Nancy Niemuth	<i>9RH</i>	<i>12/5/2000</i>
	Jennifer Holdcraft	<i>N</i>	<i>12/6/00</i>
Approved	Bill Rosebrough	<i>WRC</i>	<i>12/7/00</i>

Sent via:

Statistical Report on Clinical Signs Data from MREF Task 97-53

December 5, 2000

Overview

A total of 180 guinea pigs (GP) were administered botulinum serotype A toxin in Task 53. Clinical signs were observed for each animal during each of 28 half-day time intervals (Day 1 PM, Day 2 AM, Day 2 PM, etc. through Day 15 AM). Dosing was administered during Day 1 AM so clinical signs were not recorded for that time interval. The presence/absence of the following eleven "standard" signs were recorded in the database: appeared normal, ruffled fur, labored breathing, droopy eyelids, weak limbs, salivation, lacrimation, hindlimb paralysis at the injection site, total paralysis, other signs, and death. If "Other Signs" were checked as present, a short description of those signs was included in the comment field; these signs may have been toxin-related. A total of 38 animals were euthanized due to being in a moribund condition. For the purposes of statistical analysis, the time to euthanization was treated as the same as time to death for these animals.

Guinea pigs were treated with any of five IG (immune globulin) types. In the 12/1/00 report entitled "Statistical Analysis for MREF Task 97-53 (Final Report)", these five IG types were abbreviated as: PBIGA (primary inoculation series, lot A), PBIGB (primary inoculation series, lot B), PBIGAB (primary inoculation series, a mixture of lots A and B), BBIG (primary series plus booster inoculation, a mixture of lots A and B), and CIG (control immune globulin). The same abbreviations are used in this report.

Methods

Because the presence of one sign may mask the ability to observe another sign, statistical analyses were performed on combinations of specific signs. The combined response is defined to occur within a given time interval if any one of the signs in that combination occurred within the interval. Three combinations were defined and analyzed separately: (1) death only; (2) "severe signs", namely death, total paralysis, and/or weak limbs; and (3) any sign except normal, including other signs.

For each combined response the following endpoints were calculated for each animal: (1) incidence, that is, presence or absence of the sign during any time interval; (2) time to onset of the sign, which is the time between toxin injection and first observation of the sign; and (3) total duration of the sign within the 14-day observation period.

The response endpoints described above were computed using data pooled across specific combinations of groups. Statistical analysis was then performed on the data from these pooled groups in order to make two comparisons of interest, as described below.

- Combined data from PBIGA, PBIGB, and PBIGAB IG types (primary series) were compared to data for BBIG (primary series-plus-booster injections).

If this first comparison was not statistically significant, then the second comparison was also performed.

- Combined data from PBIGA, PBIGB, PBIGAB, and BBIG IG types (vaccinated) were compared to data for CIG (unvaccinated). The pooled group in this comparison is abbreviated as PBIGs + BBIG.

For each IG group, incidence endpoints were computed for each of two toxin dose levels, namely less than and greater than the respective LD₅₀s. The LD₅₀s used for the BBIG and CIG groups were those presented in Table 1a of the 12/1/00 statistical report (using "unadjusted" GP doses). The LD₅₀s for the pooled groups were computed by fitting a probit dose-response curve to the combined lethality data as before. The LD₅₀ for the combined PBIGA, PBIGB, and PBIGAB group data was 110,206 MIP LD₅₀ units per animal and the LD₅₀ for the combined PBIGA, PBIGB, PBIGAB, and BBIG group data was 116,489 MIP LD₅₀ units per animal. Log-likelihood tests demonstrated that no significant loss of fit resulted when the probit modeling was performed on these pooled data ($p < 0.05$).

Incidences of signs were compared between groups of animals using a two-sided Fisher's exact test conducted at the 5% significance level (SAS FREQ procedure), for the pooled IG groups described above, at each dose level.

Times to onset of signs were calculated in days to the nearest half-day. If the sign never occurred, the time to onset was right-censored at 14 days. Times to onset were modeled as a linear function of the logarithm of toxin dose using a regression procedure suitable for handling right-censored data (SAS LIFEREG procedure), with the underlying assumption that the onset times were log-normally distributed. For each IG group, the predicted time to onset at the LD₅₀ dose was estimated along with the interquartile range. A statistical hypothesis test was used to compare the mean times to onset between IG groups predicted at their respective LD₅₀ doses. Descriptive statistics (means and standard deviations) of times to onset were computed for animals that exhibited signs.

The duration of a sign was defined as the number of half-day intervals, out of a total of 28, in which the sign was noted present. If death occurred during the 14-day period, the duration of the sign was also defined to include the period of time between death and the end of the 14 days. Exploratory data analyses indicated that the dose-response curves for log dose and duration of signs were approximately sigmoidal. Therefore, duration times were modeled as a function of log dose using a generalized probit analysis (SAS GENMOD procedure). This procedure allows for sequentially repeated observations within an animal. For each IG group, the predicted duration at the LD₅₀ dose was computed along with a 95% confidence interval. A statistical hypothesis test was used to compare the predicted mean duration times for the IG groups at their respective LD₅₀ doses. Means and standard deviations of duration times were computed for animals that exhibited signs. Mean durations and model-predicted durations were converted back into units of days for reporting purposes.

Results

Table 1 displays percent incidence of severe signs and of any signs, and summarizes the results of Fisher's exact tests comparing IG groups at each dose level. None of the comparisons were statistically significant at the 5% level, indicating that there was no difference in the incidence of signs between groups at doses below or above the respective LD₅₀s for each group.

Table 2 displays total numbers of animals surviving for the entire 14-day observation period for each IG type, and the numbers of survivors showing any sign or severe signs during the last week or last day of observation. No statistical comparisons were performed on these totals. For incidence of any signs, the numbers in the last week and in the last day were virtually identical. However, the incidence of severe signs tended to be smaller during the last day than during the last week for all IG types except for the CIG group. This suggests that some of the surviving animals may have been recovering from the effects of the toxin near the end of the observation period.

Table 3 displays results of analyses of times to death. In the left half of the table, descriptive statistics are shown for each IG group. These include the average GP dose of animals that died, and the mean and standard deviation of time to death for those animals. In the right half of the table, the predicted time to onset at the GP dose LD₅₀ is presented with the interquartile range. Regression models on time to death demonstrated a strongly significant toxin dose-response effect ($p < 0.0001$) for each IG group. All regression slopes were negative, indicating that as the GP dose increases, the time to death decreases. Predicted times to death at the respective LD₅₀ doses, did not reveal significant differences between the three pooled PBIG groups versus the BBIG group, nor between the PBIGs + BBIG versus the CIG group.

Tables 4 and 5 present the results of analyses of time to onset of severe signs and time to onset of any sign, respectively, in a format similar to Table 3. For each IG group, the average dose of animals that exhibited signs is displayed, along with the mean and standard deviation of time to onset of signs for those animals. Table 4 presents descriptive statistics and results of analyses of time to onset of severe signs. Regression models on time to onset of severe signs versus log-toxin dose resulted in a strongly significant, negative dose-response slope ($p \leq 0.0001$) for each IG group. Predicted times to onset at the respective LD₅₀ doses, were not significantly different between the three pooled PBIG groups and the BBIG group. However, the predicted time was significantly less for the PBIGs + BBIG groups compared to the CIG group ($p < 0.001$), although this difference was only about 0.7 days.

Table 5 presents descriptive statistics and results of analyses of time to onset of any signs. Regression models on time to onset of any signs versus log-toxin dose resulted in a strongly significant, negative dose-response slope ($p < 0.0001$) for each IG group. The results of the pairwise group comparisons were similar to those for onset of severe signs. Predicted times to onset at the respective LD₅₀ doses, were not significantly different between the three pooled PBIG groups and the BBIG group. The predicted time was significantly less for the PBIGs + BBIG group compared to the CIG group ($p < 0.0001$), and again, this difference was about 0.7 days.

The two endpoints, duration of severe signs and duration of any sign, were highly correlated (Pearson's r correlation = 0.68, $p < 0.0001$). Therefore, statistical modeling was performed on duration of any sign, as the results for severe signs would be expected to be very similar.

Table 6 displays results of analysis of duration of any sign. For each IG group, the average dose given to animals that exhibited signs is shown (these values are identical to those displayed in Table 5), along with the mean and standard deviation of duration of signs for those animals. Model-predicted durations at the LD_{50} doses are also shown. The estimated dose-response slopes from the generalized probit models were significantly positive ($p < 0.0001$) for each IG group, indicating that as the GP dose increases, durations of signs increase. Predicted durations at the respective LD_{50} doses were not significantly different between the three pooled PBIG group and the BBIG group. The predicted duration was significantly greater for the PBIGs + BBIG versus the CIG group ($p < 0.005$).

Table 7 is a compilation of the numbers and percentages of animals for which standard signs and other signs were observed at least once, separately for each of the five IG types. "Other Signs" were grouped according to descriptions noted in the comment field of the database.

Conclusions

Statistical models and analysis performed in order to make pairwise comparisons of the IG groups with respect to the clinical signs endpoints, yielded the following conclusions:

- Incidence of signs: No statistically significant differences in the incidence of clinical signs resulted when the combined PBIG group was compared to the BBIG group, nor when the combined PBIGs + BBIG group was compared to the CIG group, at doses below or above the respective LD_{50} s for each group.
- Time to death: Predicted times to death at the respective LD_{50} doses did not show any significant differences when comparing the three combined PBIG groups to the BBIG group, nor when comparing the PBIGs + BBIG group to the CIG group.
- Time to onset of signs: The statistical analysis of time to onset of severe signs and time to onset of any signs yielded very similar results. Predicted times to onset of signs at the respective LD_{50} doses did not differ significantly when comparing the combined PBIG group to the BBIG group. The predicted time to onset of signs was significantly less for the PBIGs + BBIG group when compared to the CIG group, although this difference was less than a day.
- Duration of signs: Predicted durations of signs at the respective LD_{50} doses did not differ significantly when comparing the combined PBIG group to the BBIG group. The predicted duration of signs was significantly greater for the PBIGs + BBIG group when compared to the CIG group, and similarly, this difference was less than a day.

Table 1. Percent Incidence of Severe Signs and Any Signs for Each of Two Dose Levels within Each Pair of IG Type(s) Along with Results of Fisher's Exact Test Comparing Incidence of Signs.

Dose Level ^(a)	Death / Total Paralysis / Weak Limbs					Any Sign Except Normal				
	Percent of Animals with Severe Signs		Fisher's Exact Test P-value ^(b)	Percent of Animals with Severe Signs		Fisher's Exact Test P-value ^(c)	Percent of Animals with Any Sign		Fisher's Exact Test P-value ^(d)	Percent of Animals with Any Sign
	PBIGA, PBIGB, PBIGAB	BBIG		PBIGs + BBIG	CIG		PBIGA, PBIGB, PBIGAB	BBIG		PBIGs + BBIG
≤ LD ₅₀	76	86	0.537	79	93	0.094	81	90	0.496	84
> LD ₅₀	100	100	1.00	100	100	1.00	100	100	1.00	100

(a) ≤ LD₅₀ includes animals dosed at levels below the estimated LD₅₀
> LD₅₀ includes animals dosed at levels above the estimated LD₅₀

(b) Fisher's Exact Test for comparing incidence of severe signs between the pooled PBIG groups versus the BBIG group for the given dose level.

(c) Fisher's Exact Test for comparing incidence of severe signs between the pooled PBIG and BBIG groups versus the CIG group for the given dose level.

(d) Fisher's Exact Test for comparing incidence of any signs between the pooled PBIG groups versus the BBIG group for the given dose level.

(e) Fisher's Exact Test for comparing incidence of any signs between the pooled PBIG and BBIG groups versus the CIG group for the given dose level.

Table 2. Number of Animals Surviving for 14 Days and Incidence of Any Sign or Severe Signs Among Survivors During the Last Week and Last Day of Observation

IG Type	Number of Survivors	Any Sign Other Than Normal		Weak Limbs or Total Paralysis	
		Number in Last Week	Number on Last Day	Number in Last Week	Number on Last Day
PBIGA	21	18	18	12	9
PBIGB	21	17	17	9	8
PBIGAB	20	16	16	14	11
BBIG	20	18	16	15	9
CIG	28	25	25	25	25

Table 3. Descriptive Statistics for Times to Death for Animals that Died Only, and Predicted Times to Death Based on Regression Model Performed on Data from All Animals

Description of Comparison	IG Type(s)	Numbers of Animals		Animals that Died Only		Model-Based Results for All Animals		
		Total N	N Dead	Average Dose (MIPLD ₅₀ /Animal)	Mean Time to Death (S.D.) (Days)	LD ₅₀	Predicted Time to Death (Days) at the LD ₅₀ ^(a)	Interquartile Range
Compare IG from primary series to primary series-plus-booster injections	PBIGA, PBIGB, PBIGAB	110	48	167,800	5.0 (2.9)	110,206	13.2	5.6 - 31.1
	BBIG	30	10	178,214	5.2 (3.7)	134,540	12.2	7.1 - 21.0
Compare IG from vaccinated volunteers to control IG	PBIGs + BBIG	140	58	169,596	5.0 (3.0)	116,489	13.3	5.6 - 31.3
	CIG	40	12	16.8	8.1 (2.9)	11.4	12.9	9.6 - 17.3

- (a) Z-score test results showed that predicted times to death did not differ significantly for either pair of IG groups compared.

Table 4. Descriptive Statistics for Times to Onset of Severe Signs for Animals that Showed Severe Signs Only, and Predicted Times to Onset Based on Regression Models Performed on Data from All Animals

Description of Comparison	IG Type(s)	Number of Animals		Animals that Showed Severe Signs Only		Model-Based Results for All Animals		
		Total N	N Showing Severe Signs	Average Dose (MIPLD ₅₀ /Animal)	Mean Time to Onset (S.D.) (Days)	LD ₅₀	Predicted Time to Onset (Days) at the LD ₅₀	Interquartile Range
Compare IG from primary series to primary series-plus-booster injections	PBIGA, PBIGB, PBIGAB	110	96	133,809	1.5 (1.8)	110,206	1.2	0.9 - 1.8
	BBIG	30	27	115,254	2.1 (2.8)	134,540	1.1	0.8 - 1.4
Compare IG from vaccinated volunteers to control IG	PBIGs + BBIG	140	123	129,736	1.6 (2.1)	116,489	1.2 ^(a)	0.9 - 1.7
	CIG	40	38	9.6	3.1 (2.9)	11.4	1.9 ^(a)	1.2 - 3.1

(a) Z-score test results showed that the predicted time to onset of signs at the GP dose LD₅₀ was significantly different for the two IG groups ($p < 0.001$).

Table 5. Descriptive Statistics for Times to Onset of Any Sign for Animals that Showed Signs Only, and Predicted Times to Onset Based on Regression Models Performed on Data from All Animals

Description of Comparison	IG Type(s)	Number of Animals		Animals That Showed Signs Only		Model-Based Results for All Animals		
		Total N	N Showing Signs	Average Dose (MIPLD ₅₀ /Animal)	Mean Time to Onset (S.D.) (Days)	LD ₅₀	Predicted Time to Onset (Days) at the LD ₅₀	Interquartile Range
Compare IG from primary series to primary series-plus-booster injections	PBIGA, PBIGB, PBIGAB	110	99	130,826	1.3 (1.3)	110,206	1.1	0.8 - 1.4
	BBIG	30	28	111,144	2.1 (2.9)	134,540	1.1	0.8 - 1.3
Compare IG from vaccinated volunteers to control IG	PBIGs + BBIG	140	127	126,487	1.5 (1.8)	116,489	1.1 ^(a)	0.8 - 1.4
	CIG	40	38	9.6	2.6 (2.5)	11.4	1.8 ^(a)	1.2 - 2.7

(a) Z-score test results showed that the predicted time to onset of signs at the GP dose LD₅₀ was significantly different for the two IG groups ($p < 0.0001$).

Table 6. Descriptive Statistics for Duration of Any Sign for Animals that Showed Signs Only, and Predicted Durations Based on Regression Model Performed on Data from All Animals

Description of Comparison	IG Type(s)	Numbers of Animals		Animals that Died Only		Model-Based Results for All Animals		
		Total N	N Showing Signs	Average Dose (MIPLD ₅₀ /Animal)	Mean Time to Death (S.D.) (Days)	LD ₅₀	Predicted Time to Death (Days) at the LD ₅₀ ^(a)	Interquartile Range
Compare IG from primary series to primary series-plus-booster injections	PBIGA, PBIGB, PBIGAB	110	99	130,826	13.1 (1.8)	110,206	13.5	13.3 - 13.6
	BBIG	30	28	111,144	12.2 (3.6)	134,540	13.6	13.4 - 13.7
Compare IG from vaccinated volunteers to control IG	PBIGs + BBIG	140	127	126,487	12.9 (2.3)	116,489	13.5 ^(a)	13.4 - 13.6
	CIG	40	38	9.6	11.6 (3.1)	11.4	12.8 ^(a)	12.1 - 13.3

(a) Z-score test results showed that the predicted duration of any signs was significantly different for the two IG groups ($p < 0.005$).

Table 7. Numbers of Animals with Clinical Signs Observed for Guinea Pigs Administered Botulinum Serotype A and Treated with Any of Five IG Types ^(a)

Clinical sign	PBIGA (N=40)	PBIGB (N=30)	PBIGAB (N=40)	BBIG (N=30)	CIG (N=40)
Ruffled fur	30 (75)	15 (50)	30 (75)	17 (57)	31 (78)
Lab breathing	21 (53)	10 (33)	25 (63)	13 (43)	18 (45)
Droopy eyes	11 (28)	5 (17)	11 (28)	5 (17)	6 (15)
Weak limbs	34 (85)	24 (80)	35 (88)	26 (87)	38 (95)
Salivation	21 (53)	9 (30)	19 (48)	10 (33)	15 (38)
Lacrimation	18 (45)	5 (17)	15 (38)	5 (17)	12 (30)
Hindlimb paralysis	34 (85)	25 (83)	32 (80)	23 (77)	35 (88)
Total paralysis	4 (10)	2 (7)	3 (8)	3 (10)	4 (10)
Other signs	16 (40)	10 (33)	18 (45)	15 (50)	19 (48)
Dead	19 (48)	9 (30)	20 (50)	10 (33)	12 (30)
Other Signs ^(b) :					
Moribund/ Euthanized	11 (28)	6 (20)	13 (33)	4 (13)	4 (10)
Dehydrated	5 (13)	4 (13)	9 (23)	8 (27)	9 (23)
Diarrhea	16 (40)	8 (27)	14 (35)	12 (40)	17 (43)
Bloody diarrhea/ Bleeding from hind end	-	1 (3)	1 (3)	-	2 (5)
Hair loss	-	-	1 (3)	-	1 (3)
Prolapsed rectum/penis	1 (3)	1 (3)	-	-	2 (5)
Runny nose	-	1 (3)	-	-	-

- (a) Table entries represent the numbers of animals with the sign observed at least once, followed by the percent of animals in parentheses. The total number of animals in each IG type group is shown at the top of each column.
- (b) If multiple signs were noted in the comments, then each sign was counted individually.

Internal Distribution:

Department Files*

C. Matthews

J. Holdcraft

N. Niemuth *

B. Pierce *

RMO

* memo only

Date December 7, 2000

To Bob Hunt

From Claire Matthews CM

Subject **QA Materials for "Statistical Analysis of
Clinical Signs Data Collected in MREF Task
97-53 (Final Report)"**

S:\math\G1555\Task_53\QA-Clinical Signs Report_final.doc

Overview

The SAS system (v8) was used to process and analyze clinical signs data collected under MREF Task 97-53. Endpoints used for statistical analyses were incidence, time to onset, and durations of signs. Statistical analysis of these endpoints was conducted in order to focus on two sets of pairwise comparisons of IG groups: (1) comparing IG from primary series to primary series-plus-booster injections, and (2) comparing IG from both the primary series and primary series-plus-booster injections to the IG control group. Fisher's exact tests were used to compare incidences of signs across pairs of IG groups. Regression models suitable for handling right-censored data were used to model time to death and time to onset of signs as a function of GP dose for each pair of IG groups. These programs also estimated predicted times to death or predicted times to onset of signs that would occur at the GP dose LD₅₀s (previously reported in the 12/01/2000 report **Statistical Analysis for MREF Task 97-53 (Final Report)**, and the 12/05/2000 report **Statistical Analysis of Clinical Signs Data Collected in MREF Task 97-53 (Final Report)**). Normal z-scores were computed and used to compare the predicted times to death/onset of signs at the LD₅₀ doses across pairs of IG groups. A probit procedure which incorporated repeated observations per animal was used to model the durations of signs as a function of GP dose and to estimate predicted durations at the GP dose LD₅₀s, for each pair of IG groups. Normal z-scores were computed and used to compare the predicted duration times at the LD₅₀ doses across pairs of IG groups.

Data Inputs/Outputs

Data were entered into a Microsoft Access (V97) database by MREF technicians. The data were transferred electronically to SAS datasets using the SAS/Access software with an ODBC driver connection. The SAS program GETCLIN.SAS was used to read and merge data from the MS-Access tables tblMaster and tblClinical, store the merged data in an intermediate SAS dataset called GPCLIN.SD2, and to print out raw data listings of clinical signs for QA audits. The SAS datasets GPDATAALL.SD2 and GPPOTALL.SD2 were previously created by the program GETGP.SAS (as described in **QA Materials for Statistical Analysis for MREF Task 97-53 Final Report**); they contain GP dose data and potency data for each animal. The SAS program CRTCLIN.SAS was used to read in the intermediate SAS datasets GPCLIN.SD2 and GPDATAALL.SD2, and to create a merged dataset called GPCLIN1.SD2, which was used in the statistical analysis of incidence, time to onset, and duration of signs. Finally, the SAS program GETCOMM.SAS was used to read in clinical signs description data from the MS-Access table tblClinical and combined with GPPOTALL.SD2 created the clinical comment dataset CLINCOMM.SD2.

Performance Test Methods/Results

The transfer of potency experiment dosing data and of raw clinical signs data, was tested by listing the SAS datasets GPDATA.LLSD2 and GPCLIN.SD2 and sending hard copies to the MREF for quality assurance review.

The SAS program PROBSEP1.SAS was used to fit a dose-response probit model to pooled potency data in order to estimate the LD₅₀ from these pooled IG groups. The SAS programs CLINFISH.SAS, CLINSURV.SAS, LIFETTD.SAS, GENMODRN.SAS, and FREQCOMM.SAS were used for performing data compilation, modeling and analyses to produce the results reported in Tables 1 through 7. The SAS program CORRTIME.SAS was also used to compute correlation coefficients quoted in the text of the clinical signs report. A listing of each program and its output is attached. Previously-calculated GP dose LD₅₀s for each IG group were stored in a SAS dataset called PCTSEP1.SD2 which was read into the SAS programs CLINFISH.SAS, LIFETTD.SAS, and GENMODRN.SAS.

Probit dose-response models were fitted to pooled data in the program PROBSEP1.SAS using the SAS PROBIT procedure. Fisher's exact tests were computed in the program CLINFISH.SAS using the SAS FREQ procedure. No performance tests of the SAS FREQ and PROBIT procedures are required.

Right-censored regression models were fitted in the program LIFETTD.SAS using the SAS LIFEREG procedure, which calculates predicted values and interquartile ranges. No performance tests of the LIFEREG procedure calculations are required. Normal z-scores were calculated as the difference between predicted log-times to onset divided by the standard error of the difference. The p-values for these test statistics were derived using the SAS probability PROBNORM function. PROBNORM is a built-in SAS library function and does not require performance testing.

Probit models suitable for handling repeated observations data were fitted in the program GENMODRN.SAS using the SAS GENMOD procedure, which calculates predicted values and 95% confidence intervals. No performance tests of the GENMOD procedure calculations are required. Normal z-scores were calculated as the difference between predicted log-duration times divided by the standard error of the difference. As in the LIFETTD.SAS program, the p-values for these test statistics were derived using the SAS probability PROBNORM function.

Simple descriptive statistics such average times to death for animals that died, and average times to onset of signs and durations of signs for animals with signs, were calculated in the programs LIFETTD.SAS and GENMODRN.SAS using the SAS MEANS procedure. Incidences of signs were also computed using the SAS MEANS procedures in the programs CLINFISH.SAS, CLINSURV.SAS, and FREQCOMM.SAS. No performance tests of the SAS MEANS procedure calculations are required. Pearson's r correlation coefficients reported in the Task 53 report text were calculated using the SAS CORR procedure. No performance tests of the SAS CORR procedure calculations are required.

M. Claire Matthews
RESPONSIBLE INDIVIDUAL

Nancy A. Niemuth
RESPONSIBLE INDIVIDUAL

MCM:llj

Robert E. Shum
STUDY DIRECTOR

For Review and Approval

	Name	Internal	Date
Originator	C. Matthews	CM	12/7/00
Concurrence	N. Niemuth	N	12/7/00
	J. Holdcraft	JRH	12/7/00
Approved	B. Pierce	WPR	12/7/00

LIBNAME SASDB 'D:\TASK53';
OPTIONS LS=168 PS=59;

* GETCLIN.SAS; * read clinical signs data from Access database,
print raw data listing for QA;

%MACRO GETCLIN(INMDB);

proc sql feedback;

connect to odbc as &INMDB (dsn=&INMDB uid="matthews" pwd="task51pw" log);

CREATE TABLE MAST AS (SELECT *

from connection to &INMDB (select STUDYNUM,
EXPNUM,
STUDYDATE AS EXPDATE,
ASSAYTYPE AS ASSAYTYP,
SEROTYPE
from tblMaster));

quit;

proc sql feedback;

connect to odbc as &INMDB (dsn=&INMDB uid="matthews" pwd="task51pw" log);

CREATE TABLE CLINPUT AS (SELECT *

from connection to &INMDB(select
STUDYDATE AS SDATE,
EXPNUM,
GPID,
CLINICALDATE AS CDATE,
AMORPM AS AMPM,
NORMAL,
RUFFLED FUR AS RUFFLED,
LABORED BREATHING AS LABREATH,
DEAD,
DROOPYEYES AS DROOPEYE,
WEAK LIMBS AS WEAK LIMB,
OTHER SYMPTOM AS OTHERSYM,
SALIVATION AS SALIVATN,
LACRIMATION AS LACRIMAT,
HIND LIMB PARALYSIS AS HIND LIMB,
TOTAL PARALYSIS AS TOTPARAL,
clinicalcomment as comment
from tblClinical));

disconnect from &INMDB;

quit;

%MEND;

%GETCLIN(TASK9753);

DATA MAST2; SET MAST;

FORMAT STUDYNUM;

IF UPCASE(COMPRESS(STUDYNUM, '-')) = 'G155553A';

DATA CLIN; SET CLINPUT;

date = datepart(sdate);

clindate = datepart(cdate);

FORMAT NORMAL RUFFLED LABREATH DEAD DROOPEYE WEAK LIMB OTHERSYM TOTPARAL

SALIVATN LACRIMAT HIND LIMB;

format date clindate mmddyy8.;

FORMAT GPID COMMENT;

drop sdate cdate;

RUN;

PROC SORT DATA=MAST2;

BY EXPNUM;

PROC SORT DATA=CLIN;

BY EXPNUM GPID CLINDATE AMPM;

DATA CLIN2; MERGE MAST2 CLIN (IN=IN2);

BY EXPNUM;

IF IN2 OR ASSAYTYP = 'P';

format serotype EXPNUM;

PROC SORT DATA=CLIN2;

BY DATE EXPNUM GPID CLINDATE AMPM;

SAS program to read Access
database, create raw SAS
dataset of clinical signs +
create listings for QA as
contained in

" Task 97-53 Raw Data
Listing of Guinea Pig Clinical
Observations (Revised) "
dated 9/14/2000

Jennifer Holdcraft
12/5/2000

*****;

%MACRO OUTSIGN(SIGN, SIGNNAME);

TOT = 0;

DO I=1 TO 29;

IF &SIGN.S{I} = 0 THEN XS{I} = '-';

IF &SIGN.S{I} > 0 THEN DO;

TOT = TOT + 1;

XS{I} = '0'; *alt-251;

END;

END;

CLINSIGN = "&SIGNNAME";

IF TOT > 0 THEN OUTPUT;

%MEND;

OPTIONS MPRINT SYMBOLGEN;

DATA D1; SET CLIN2;

BY GPID NOTSORTED;

LENGTH CLINSIGN \$20;

RETAIN NSEQ N1-N29 R1-R29 B1-B29 D1-D29 E1-E29 W1-W29 O1-O29

S1-S29 L1-L29 H1-H29 T1-T29 SUM1-SUM29;

RETAIN ISTART;

ARRAY NS{29} N1-N29;

ARRAY RS{29} R1-R29;

ARRAY BS{29} B1-B29;

ARRAY DS{29} D1-D29;

ARRAY ES{29} E1-E29;

ARRAY WS{29} W1-W29;

ARRAY OS{29} O1-O29;

ARRAY SS{29} S1-S29;

ARRAY LS{29} L1-L29;

ARRAY HS{29} H1-H29;

ARRAY TS{29} T1-T29;

ARRAY SUMS{29} SUM1-SUM29;

get index of first reading time where at least one box is checked (1 or 2);

TEMPSUM = SUM(NORMAL, RUFFLED, LABREATH, DEAD, DROOPEYE, WEAKLIMB, OTHERSYM,

SALIVATN, LACRIMAT, HINDLIMB, TOTPARAL);

IF FIRST.GPID THEN DO;

NSEQ=1;

IF AMPM = 'PM' THEN NSEQ=2;

ISTART = NSEQ;

IF AMPM = 'AM' AND TEMPSUM LE 0 THEN ISTART = 2;

DO I=1 TO 29;

NS{I} = .;

RS{I} = .;

BS{I} = .;

DS{I} = .;

ES{I} = .;

WS{I} = .;

OS{I} = .;

SS{I} = .;

LS{I} = .;

HS{I} = .;

TS{I} = .;

SUMS{I} = .;

END;

END;

ELSE NSEQ = NSEQ+1;

NS{NSEQ} = NORMAL;

RS{NSEQ} = RUFFLED;

BS{NSEQ} = LABREATH;

DS{NSEQ} = DEAD;

ES{NSEQ} = DROOPEYE;

WS{NSEQ} = WEAKLIMB;

OS{NSEQ} = OTHERSYM;

SS{NSEQ} = SALIVATN;

```

LS{NSEQ} = LACRIMAT;
HS{NSEQ} = HINDLIMB;
TS{NSEQ} = TOTPARAL;
SUMS{NSEQ} = TEMPSUM;
IF LAST.GPID;

```

```

DROP I NORMAL--TOTPARAL TEMPSUM;
DROP NSEQ N1-N29 R1-R29 B1-B29 D1-D29 E1-E29 W1-W29 O1-O29
      S1-S29 L1-L29 H1-H29 T1-T29 TOT;
DROP CLINDATE AMPM;
ARRAY XS{29} $ X1-X29;
DO I=1 TO 29;
  XS{I} = ' ';
END;

```

```

%OUTSIGN(N, Normal);
%OUTSIGN(R, Ruffled fur );
%OUTSIGN(B, Lab breathing);
%OUTSIGN(E, Droopy eyes );
%OUTSIGN(W, Weak limbs );
%OUTSIGN(O, Other signs );
%OUTSIGN(S, Salivation );
%OUTSIGN(L, Lacrimation );
%OUTSIGN(H, Hindlimb paralysis);
%OUTSIGN(T, Total paralysis);
%OUTSIGN(D, Dead );

```

```

PROC SORT DATA=D1;
  BY DATE EXPNUM GPID ;

```

```

DATA D2; SET D1;
  ARRAY XS{29} $ X1-X29;
  ARRAY OUTXS{15} $ OX1-OX15;
  ARRAY SUMS{29} SUM1-SUM29;

```

```

DO I = 1 TO 14;
  I1 = I*2-1;
  I2 = I*2;
  OUTXS{I} = TRIM(XS{I1}) || ' ' || TRIM(XS{I2});
  IF I=1 AND XS{I} = ' ' THEN OUTXS{I} = '- ' || TRIM(XS{I2});
END;
OUTXS{15} = TRIM(XS{29}) || ' ';

```

```

LABEL OX1 = 'Day 1*AM PM';
LABEL OX2 = 'Day 2*AM PM';
LABEL OX3 = 'Day 3*AM PM';
LABEL OX4 = 'Day 4*AM PM';
LABEL OX5 = 'Day 5*AM PM';
LABEL OX6 = 'Day 6*AM PM';
LABEL OX7 = 'Day 7*AM PM';
LABEL OX8 = 'Day 8*AM PM';
LABEL OX9 = 'Day 9*AM PM';
LABEL OX10 = 'Day 10*AM PM';
LABEL OX11 = 'Day 11*AM PM';
LABEL OX12 = 'Day 12*AM PM';
LABEL OX13 = 'Day 13*AM PM';
LABEL OX14 = 'Day 14*AM PM';
LABEL OX15 = 'Day 15*AM';

```

```

ARRAY SS{29} S1-S29;
DO I=1 TO 29;
  SS{I} = .;
  IF XS{I} = '-' THEN DO;
    SS{I} = 0;
    IF SUMS{I} LE 0 THEN SS{I} = .; * no observation taken, all signs are missing;
  END;
  ELSE IF XS{I} = '0' THEN SS{I} = 1;
END;
IF ISTART = 2 THEN S1 = .;

```

```

GPNO = 1*SUBSTR(GPID,1,3);

```

```

PROC SORT DATA=D2;

```

BY DATE SEROTYPE EXPNUM GPNO;

* listing for Q.A.;

OPTIONS LS=172 PS=59 PAGENO=1;

NOO PRINT DATA=D2 SPLIT='*';

BY DATE SEROTYPE EXPNUM GPID NOTSORTED;

ID DATE SEROTYPE EXPNUM GPID ;

VAR CLINSIGN OX1-OX15;

TITLE1 'Study No. G1555-53A';

TITLE2 'TASK 97-53 POTENCY EXPERIMENTS - LISTING OF OBSERVED GUINEA PIG CLINICAL SIGNS';

TITLE3 ' ';

LABEL SEROTYPE = 'Sero-*type';

label date = 'Date';

label expnum = 'Expm*No.';

label gpid = 'GPig*ID';

LABEL CLINSIGN = 'Clinical*Sign';

RUN;

* save SAS dataset version of QA listing - variable number of records per animal;

DATA SASDB.GPCLIN; SET D2;

KEEP DATE SEROTYPE EXPNUM GPID CLINSIGN S1-S29;

RUN;□

LIBNAME SASDB 'D:\TASK53';
OPTIONS LS=172 PS=59;

SAS program to create statistical dataset of clinical signs for analyzing times to onset

Jennifer Holdcraft

12/5/2000

* CRTCLIN.SAS; * first run Getclin.SAS and Getgp.SAS, then this program -
creates a working SAS dataset with a fixed no. of records per
animal, suitable for statistical analyses. Also contains
index variables for subscript of boxes checked to time to
death, time to onset of severe signs (death, WL, TP),
and time to onset of any sign;

* Get guinea pig dataset created by Getgp.Sas - used to produce QA listings;

DATA GPOT; SET SASDB.GPDATAALL (KEEP= SEROTYPE DATE EXPNUM GPID GPDEATH
IGTYPE RUN DOSEDIL LD50ML LD50ML2 GPDOSE GPDOSE2 PCRT);

PROC SORT DATA=GPOT;
BY EXPNUM GPID;
* BY SEROTYPE RUN IGTYPE EXPNUM GPID;
RUN;

*****;

* get clinical signs data read by Getclin.sas - also used to produce QA listings;

DATA CLIN1; SET SASDB.GPCLIN (RENAME=(GPID=TEMPGP));
GPID = 1*TEMPGP; DROP TEMPGP;

LENGTH SIGN1-SIGN11 \$20;
ARRAY SIGNS {11} \$ SIGN1-SIGN11
('Normal', 'Ruffled fur ', 'Lab breathing', 'Droopy eyes', 'Weak limbs',
'Salivation', 'Lacrimation', 'Hindlimb paralysis', 'Total paralysis', 'Other signs', 'Dead');

DO I=1 TO 11;
IF CLINSIGN = SIGNS{I} THEN SIGNNO=I;
END;
DROP I SIGN1-SIGN11;

ROC SORT DATA=CLIN1;
BY EXPNUM GPID SIGNNO ;

*****;

* create 11 records for each GP;
DATA MATRIX; SET CLIN1;
BY GPID NOTSORTED;
IF LAST.GPID;
KEEP EXPNUM GPID SEROTYPE DATE;

DATA MATRIX2; SET MATRIX;
LENGTH SIGN1-SIGN11 \$20;
ARRAY SIGNS {11} \$ SIGN1-SIGN11
('Normal', 'Ruffled fur ', 'Lab breathing', 'Droopy eyes', 'Weak limbs',
'Salivation', 'Lacrimation', 'Hindlimb paralysis', 'Total paralysis', 'Other signs', 'Dead');

DO I=1 TO 11;
CLINSIGN = SIGNS{I};
SIGNNO=I;
OUTPUT;
END;
DROP I SIGN1-SIGN11;

* end up with dataset with 11 records per animal;

DATA CLIN2; MERGE CLIN1 (IN=IN1) MATRIX2 (IN=IN2);
BY EXPNUM GPID SIGNNO;

* merge with GP information;

DATA CLIN3;
LENGTH DATE 8. SEROTYPE \$1 IGTYPE \$10 EXPNUM \$5 GPID 8.
DOSEDIL GPDOSE GPDOSE2 8.;
MERGE CLIN2 GPOT (DROP=GPDEATH);
BY EXPNUM GPID;

*← program is attached
in " QA materials for
Statistical Analysis for
MREF Task 97-53 (Final Report) "*

```

* get index of time of death and merge with all records;
DATA DTH; SET CLIN3;
  IF CLINSIGN = 'Dead';
  INDTH = 30;
  ARRAY SS{29} S1-S29;
  DO I=1 TO 29;
    IF SS{I} = 1 THEN INDTH = I;
  END;
  DROP S1-S29 I;
  KEEP SEROTYPE EXPNUM GPID INDTH;

```

```

PROC MEANS NOPRINT DATA=CLIN3;
  BY EXPNUM GPID;
  VAR S1-S29;
  OUTPUT OUT=SUMOUT (DROP=_TYPE_ _FREQ_) SUM=SUM1-SUM29;

```

* If any sign was recorded in each of the 29 boxes, including normal, change missing values to zeroes;

```

DATA CLIN4; MERGE CLIN3 DTH SUMOUT;
  BY EXPNUM GPID;
  ARRAY SS{29} S1-S29;
  ARRAY SUMS{29} SUM1-SUM29;
  DO I=1 TO 29;
    IF SUMS{I} GE 1 AND SS{I} = . THEN SS{I} = 0;
  END;
  DROP SUM1-SUM29 I;

```

```

* get index for time to onset of any signs;
PROC MEANS NOPRINT DATA=CLIN4;
  WHERE SIGNNO > 1;
  BY EXPNUM GPID;
  VAR S1-S29;
  OUTPUT OUT=SUMOUT2 (DROP=_TYPE_ _FREQ_) SUM=SUM1-SUM29;

```

```

DATA DIND2; SET SUMOUT2;
  ARRAY SUMS{29} SUM1-SUM29;
  DO I=1 TO 29;
    IF SUMS{I} GE 1 THEN DO;
      INDANY = I;
      GO TO JUMP1;
    END;
  END;
  JUMP1: DROP SUM1-SUM29 I;
  KEEP EXPNUM GPID INDANY;

```

```

* get index for time to onset of severe signs (weak limbs, tot paral, death);
PROC MEANS NOPRINT DATA=CLIN4;
  WHERE SIGNNO IN (5, 9, 11);
  BY EXPNUM GPID;
  VAR S1-S29;
  OUTPUT OUT=SUMOUT3 (DROP=_TYPE_ _FREQ_) SUM=SUM1-SUM29;

```

```

DATA DIND3; SET SUMOUT3;
  ARRAY SUMS{29} SUM1-SUM29;
  DO I=1 TO 29;
    IF SUMS{I} GE 1 THEN DO;
      INDSEV = I;
      GO TO JUMP1;
    END;
  END;
  JUMP1: DROP SUM1-SUM29 I;
  KEEP EXPNUM GPID INDSEV;

```

```

DATA TEMPCLIN; MERGE CLIN4 DIND2 DIND3;
  BY EXPNUM GPID;
  IF INDANY=. THEN INDANY=30;
  IF INDSEV=. THEN INDSEV=30;
  RUN;

```

```

DATA SASDB.GPCLIN1; SET TEMPCLIN (RENAME=(EXPNUM=TEMPNUM));
  EXPNUM = 1*TEMPNUM;

```

DROP TEMPNUM;

PROC SORT DATA=SASDB.GPCLIN1;

BY SEROTYPE IGTYPE EXPNUM GPID SIGNNO;

PTIONS LS=169 PS=65 PAGENO=1;

PROC PRINT DATA=SASDB.GPCLIN1 (OBS=240);

BY SEROTYPE IGTYPE DATE EXPNUM GPID GPD0SE GPD0SE2 NOTSORTED;

ID SEROTYPE IGTYPE DATE EXPNUM GPID GPD0SE GPD0SE2;

VAR SIGNNO CLINSIGN S1-S29 INDTH INDANY INDSEV;

FORMAT EXPNUM 6.0 GPID 5.0 GPD0SE GPD0SE2 10.1 SIGNNO S1 INDTH 4.0;

FORMAT SEROTYPE \$CHAR4. IGTYPE \$CHAR8.;

FORMAT LD50ML COMMA8.0;

TITLE 'TASK 53 GPCLIN1.SD2';

RUN;

□

LIBNAME SASDB 'D:\TASK53';
OPTIONS LS=168 PS=59;

* GETCOMM.SAS;

SAS program to create clinical signs
description dataset Clincomm.sds

Jennifer R Holdcraft
12/5/2000

```
%MACRO GETCLIN(INMDB);

proc sql feedback;

proc sql feedback;
  connect to odbc as &INMDB (dsn=&INMDB uid="matthews" pwd="task51pw" log);
  CREATE TABLE MAST AS (SELECT *
    from connection to &INMDB (select EXPNUM,
                                   SEROTYPE
    from tblMaster ));

quit;

proc sql feedback;
  connect to odbc as &INMDB (dsn=&INMDB uid="matthews" pwd="task51pw" log);
  CREATE TABLE CLINPUT AS ( SELECT *
    from connection to &INMDB(select
                                   STUDYDATE      AS SDATE,
                                   EXPNUM,
                                   GPID,
                                   CLINICALDATE    AS CDATE,
                                   AMORPM          AS AMPM,
                                   NORMAL,
                                   RUFFLEDUR       AS RUFFLED,
                                   LABOREDBREATHING AS LABREATH,
                                   DEAD,
                                   DROOPYEYES      AS DROOPEYE,
                                   WEAKLIMBS       AS WEAKLIMB,
                                   OTHERSYMPTOM    AS OTHERSYM,
                                   TOTALPARALYSIS  AS TOTPARAL,
                                   clinicalcomment as comment
    from tblClinical ));

  disconnect from &INMDB;
quit;
%mend;
%GETCLIN(TASK9753 );

DATA CLINO; SET CLINPUT (RENAME=GPID=TEMPID);
  GPID = 1*TEMPID; DROP TEMPID;
  date      = datepart(sdate);
  clindate  = datepart(cdate);
  FORMAT NORMAL RUFFLED LABREATH DEAD DROOPEYE WEAKLIMB OTHERSYM TOTPARAL;
  format date clindate mmddyy8.; FORMAT GPID ;
  COMMENT = LEFT(COMMENT);
  IF OTHERSYM = 1 OR COMMENT NE ' ';
  drop sdate cdate;

PROC SORT DATA=MAST;
  BY EXPNUM;

PROC SORT DATA=CLINO;
  BY EXPNUM GPID CLINDATE AMPM;

DATA CLIN1; MERGE MAST CLINO (IN=IN2);
  BY EXPNUM;
  IF IN2;
  format serotype;

PROC SORT DATA=CLIN1;
  BY GPID;

* get guinea pig dataset created by Getgp.Sas - used to produce QA listings;

DATA GPOT; SET SASDB.GPPOTALL (KEEP= GPID IGTYP GPDOSE);

PROC SORT DATA=GPOT;
  BY GPID;
RUN;
```

← program listed in
"QA materials for Statistical
Analysis for MREF Task 97-53
(Final Report)"

```

DATA CLIN2; MERGE CLIN1(IN=IN1) GPOT(IN=IN2);
  BY GPID;
  IF IN1;
  DATETIME = PUT(CLINDATE,MMDDYY8.) || ' ' || TRIM(AMPM);
  IF OTHERSYM = 1 THEN OTHER = '0'; *alt-251;
  ELSE OTHER = '-';
  FORMAT EXPNUM;

PROC SORT DATA=CLIN2;
  BY DATE EXPNUM GPID CLINDATE AMPM;

/*
OPTIONS LS=170 PS=59 PAGENO=1;
PROC PRINT DATA=CLIN2 (OBS=200);
  BY SEROTYPE DATE EXPNUM IGTYPE GPID NOTSORTED;
  ID SEROTYPE DATE EXPNUM IGTYPE GPID ;
  VAR CLINDATE AMPM NORMAL--TOTPARAL;
TITLE 'PHASES 2 & 3 POTENCY EXPERIMENTS - CLINICAL OBSERVS ON GUINEA PIGS';
RUN;

PROC FREQ; TABLES COMMENT; RUN;
*/

* listing for Q.A.;

OPTIONS LS=120 PS=78 PAGENO=1;
PROC PRINT DATA=CLIN2 SPLIT='*';
  BY DATE EXPNUM GPID NOTSORTED;
  ID DATE EXPNUM GPID ;
  VAR DATETIME OTHER COMMENT;
  FORMAT COMMENT $CHAR55.;
  TITLE1 'Study No. G1555-53A';
  TITLE2 'TASK 97-53 POTENCY EXPERIMENTS - GUINEA PIG CLINICAL SIGNS';
  TITLE3 'LISTING OF COMMENTS DESCRIBING OTHER SYMPTOMS OBSERVED';
  TITLE4 ' ';
  label expnum = 'Experiment*Number';
  label date = 'Expm*Start Date';
  label datetime = 'Date, Time*Observed';
  label expnum = 'Exp*No.';
  label gpig = 'GPig*ID';
  label other = 'Other*Symptoms';
  label comment = 'Comment';
RUN;

DATA SASDB.CLINCOMM; SET CLIN2;
RUN;

*****;

/*
DATA CHK; SET sasdb.CLINcomm (KEEP=GPID COMMENT);
  IF COMMENT NE ' ';
  COMMENT = UPCASE(LEFT(COMMENT));

PROC SORT DATA=CHK;
  BY GPID COMMENT;

DATA CHK2; SET CHK;
  BY GPID COMMENT;
  IF LAST.GPID OR LAST.COMMENT;

OPTIONS LS=106 PS=78 PAGENO=1;
PROC FREQ DATA=CHK;
  TABLES COMMENT;
TITLE 'TASK 51 CLINICAL SIGNS - OTHER SYMPTOMS';
*/
RUN;

```

```

/*****
**** Program Name Probsep1.sas
**** Purpose: probit analyses on potency data for each IGtype
**** Project Number:
**** Date Written:
**** Last Update: 11/29/2000
**** Data Sets Used:
**** Data Sets Created:
**** Files Used:
**** Files Created:
**** Comments: makes tables 1a and 1b of final report
**** Programmer: Claire Matthews, Jennifer R. Holdcraft
*****/
libname jenndata '\\pc-holdcraft\d\my projects\mref\task53\sas datasets';
libname jennres '\\pc-holdcraft\d\my projects\mref\task53\results';
libname sas53 '\\ns-bco-fs3\sdasdata\mref\task53';
options ls=120 ps=78;

```

SAS program to get LD50s
for groups analyzed in
pairwise comparisons of
clinical signs later (some
IG types are pooled)

Jennifer Holdcraft
12/5/2000

```

* probsep1.sas; * probit analyses on potency data for each IGtype;

data d1a; length igtype $12;
  set sas53.gpdata11 (keep=assaytyp expnum serotype date igtype gpiddose gpdeath);
  if assaytyp = 'P';
  task = 53;
  if igtype = 'PBIGA' then igno = 1;
  if igtype = 'PBIGB' then igno = 2;
  if igtype = 'PBIGAB' then igno = 3;
  if igtype = 'BBIG' then igno = 4;
  if igtype = 'CIG' then igno = 5;
run;

data d1b; length igtype $12;
  set sas53.gp_pot52
  (keep=assaytyp serotype expnum date antibody gpiddose gpdeath rename=(antibody=igtype));
  if serotype = 'A';
  gpdeath = substr(gpdeath,1,1);
  task = 52;
  if igtype = 'BIG' then do; igtype = '52-BIG'; igno=0.1; end;
  if igtype = 'VIG' then do; igtype = '52-VIG'; igno=0.2; end;
run;

data d1c;
  set d1a;
  if igno le 3;
  igtype = '3PBIGS';
  igno = 11;
run;

data d1d;
  set d1a;
  if igno le 4;
  igtype = 'PBIGS+BBIG';
  igno = 12;
run;

data d1; length igtype $12;
  set d1a d1b d1c d1d;
  gpdeath = upcase(gpdeath);
  if gpdeath='Y' then ndead=1;
  if gpdeath='N' then ndead=0;
  * if task=53 then gpdose = gpdose2;
  if gpdose > 0 then logdose = log10(gpdose);
  n=1;
run;

title1 'Study No. G1555-53A - use unadjusted GP doses';
* title1 'Study No. G1555-53A - use adjusted GP doses for 2 experiments #91004-5';

proc sort data=d1; by serotype igno igtype; run;

proc means data=d1 noprint;
  by serotype igno igtype;
  where gpdose > 0 and ndead ne .;

```

```

var n;
output out=nout (drop=_type_ _freq_) sum=nanims;
run;

data d1hyp;
  set d1 (keep=serotype igtype igno);
  by serotype igno igtype;
  if first.igtype;
run;

data d1hyp;
  set d1hyp;
  ipred=1;
  n=1; ndead=.;
  do logdose=-.8 to 6.8 by .1; gpdose = 10**logdose; output; end;
run;

data d2;
  set d1 d1hyp;
run;

proc sort data=d2; by serotype igno igtype; run;

options ls=122 ps=78 pageno=1;
proc probit log10 data=d2 covout outest=outest1;
  by serotype igno igtype;
  model ndead/n = gpdose /d=normal inversecl;
  output out=predout p = predval;
  title2 "Separate slopes model of pct dead versus log of GPdose for each IG type";
run;

data jennndata.predout; *data jennndata.predouta;
  set predout;
run;

data predout2; set predout;
  if ipred=1 then predpct = predval;
  else pctdead = ndead/n;
  if igtype = 'CIG' then plotsym = 'O';
  if igtype = 'BBIG' then plotsym = '1';
  if igtype = 'PBIGA' then plotsym = 'a';
  if igtype = 'PBIGB' then plotsym = 'b';
  if igtype = 'PBIGAB' then plotsym = 'c';
  if igtype = '52-BIG' then plotsym = 'B';
  if igtype = '52-VIG' then plotsym = 'V';
run;

options ls=170 ps=59 pageno=1;
proc plot nolegend data=predout2;
  where igno < 10;
  plot predpct * logdose = plotsym; *'. ' ;
  title2 "Plot of predicted percent dead from separate slopes model for each IGtype";
  title3 'Symbols: O=CIG, 1=BBIG, a=PBIGA, b=PBIGB, c=PBIGAC, B=Task 52 BIG, V = Task 52 VIG';
  label predpct = 'Predicted Percent Dead';
run; quit;

proc sort data=predout2; by igtype; run;

options ls=170 ps=59 pageno=1;
proc plot nolegend data=predout2;
  by igtype;
  plot predpct * logdose = plotsym pctdead*logdose = 'x'/overlay;
  title2 "Plot of predicted percent dead from separate slopes model for each IGtype";
run; quit;

*****;
* Get file of estimated coefficients and covariance matrix written *;
* by Proc NLin, pull off appropriate values and collapse down to a *;
  single record for each group (response) *;
*****;

data outn1;
  set outest1;

```

```

by serotype igno igtype;
retain slp int1 varb1 covb0b1 varb0 lnlike;
if (_type_='PARMS') then do;
    slp = gpdose;
    int1 = intercept;
end;

else if (_type_='COV' and _name_='GPDSE') then do;
    varb1 = gpdose;
    covb0b1 = intercept;
end;

else if (_type_='COV' and _name_='Intercept') then do;
    varb0 = intercept;
end;
lnlike = _lnlike_;
if last.igtype;

logld50 = -(int1 - probit(.5))/slp;
ld50 = 10**logld50;
keep serotype igtype igno slp int1 varb1 covb0b1 varb0 lnlike ld50;
run;

data estsep1; set outn1; run;
/*
proc print noobs data=estsep1;
by serotype;
title4 'output coefficients and covariance matrix from probit regression';
*/

data jenndata.estsep1; *data jenndata.estsep1a; set estsep1; run;

/*
* see if 3 pbigs can be combined;
ita combest; set sasdb.estsep1 (in=ins keep=serotype igtype igno lnlike);
if igno in (1,2,3) then regtype=1;
else if igno eq 11 then regtype=2;

* see if 3 pbigs + bbig can be combined;
* if igno in (1,2,3,4) then regtype=1;
* else if igno eq 12 then regtype=2;
else delete;

data combest2; set combest;
by serotype regtype;
retain septot3 septot1;
if first.serotype then septot3 = 0;
if regtype=1 then septot3 = septot3 + lnlike;
if regtype=2 then septot1 = lnlike;
if last.serotype then do;
    chisq = 2*(septot3 - septot1);
    df = 4; *df=6;
    pvalue = 1 - probchi(chisq, df);
end;

options ls=92 ps=62;
proc print noobs data=combest2;
by serotype;
title2 'See if 3 PBIGs can be combined into a single probit curve';
*title2 'See if 3 PBIGs + BBIG can be combined into a single probit curve';
run;
*/

*****;
* Generate percentiles to be estimated from the probit equation *;
*****;

data outpct;
set jenndata.estsep1; *set jenndata.estsep1a;
pctile = 50;

```

```

run;
/*
* do pctlile= 1, 5, 10, 16, 20, 25, 30, 40, 50, 60, 70, 75, 80, 84, 90, 95, 99;

do pctlile = .001 to .009 by .001;
  output;
end;
do pctlile = .01 to .9 by .01;
  output;
end;
do pctlile = 1 to 99 by 1;
  output;
end;
do pctlile = 99.1 to 99.9 by .1;
  output;
end;
do pctlile = 99.91 to 99.99 by .01;
  output;
end;
*/

data outpct2;
  set outpct;
  t = 1.96;
  * t = 1.645; * 90% c.i.;
  prp = pctlile/100;
  probt=probit(prp);
  if (varb0 gt 0 and varb1 gt 0) then do;
    seb0 = sqrt(varb0);
    seb1 = sqrt(varb1);
    corrb0b1 = covb0b1/sqrt(varb0*varb1);
  end;
  if (slp ne 0 and slp ne .) then do;
    logldpct = -(int1 - probt)/slp;
    varx = (1/slp**2)*(seb0**2) + ((int1-probt)**2/slp**4)*(seb1**2)
      - 2*((int1-probt)/slp**3)*seb0*seb1*corrb0b1;
    sdx = sqrt(varx);
  end;
  a0 = int1 - probt;
  aa = slp*slp - t*t*varb1;
  bb = a0 *slp - t*t*covb0b1;
  cc = a0 *a0 - t*t*varb0;
  delta = .000001;
  quad = bb*bb - aa*cc;
  length comment $9;
  if (quad le delta and quad ne .) then do;
    loglcb=-100;
    logucb=100;
  end;
  else if (quad gt delta) then do;
    if (aa gt delta) then do;
      loglcb = (-bb - sqrt(quad))/aa;
      logucb = (-bb + sqrt(quad))/aa;
    end;
  else if (aa lt -1*delta and aa ne .) then do;
    loglcb = (-bb - sqrt(quad))/aa;
    logucb = (-bb + sqrt(quad))/aa;
    comment='???';
  end;
  else if (abs(aa) le delta and aa ne .) then do;
    thetas = -cc/(2*bb);
    if (logldpct lt thetas) then do;
      loglcb = -100;
      logucb = thetas;
    end;
  else if (logldpct ge thetas) then do;
    loglcb = thetas;
    logucb = 100;
  end;
end;
end;
end;

```

```

if (logldpct ne . and loglcb ne . and logucb ne .) then do;
if (abs(logldpct) lt 30) then ldpct = (10**(logldpct));
else if (logldpct lt -30) then ldpct = -999999;
else if (logldpct gt 30) then ldpct = 999999;
if (abs(loglcb) lt 30) then lcb = (10**(loglcb));
else if (loglcb lt -30) then lcb = -999999;
else if (loglcb gt 30) then lcb = 999999;
if (abs(logucb) lt 30) then ucb = (10**(logucb));
else if (logucb lt -30) then ucb = -999999;
else if (logucb gt 30) then ucb = 999999;
end;

length fiellcb $24.;
fiellcb = '(' || put(lcb,comma9.2) || ', ' || put(ucb,comma10.2) || ')';
if comment ne ' ' then fiellcb = trim(fiellcb) || ' ' || trim(comment);

loglcbd = logldpct - 1.96*sd;
logucbd = logldpct + 1.96*sd;
lcbd = (10**(loglcbd));
ucbd = (10**(logucbd));
deltacb = '(' || put(lcbd,comma9.2) || ', ' || put(ucbd,comma10.2) || ')';
label deltabc = 'Delta*Conf.Bounds';
label pctile = 'Perc-*entile';
label probt = 'Probit of*Percentile';
label logldpct = 'Log(Eff. Dose)*for Percentile';
label ldpct = 'Effective Dose*for Percentile';
label sd = 'Std. Error of*Log(Eff. Dose)';
label lcb = 'Lower Confid-* ence Bound';
label ucb = 'Upper Confid-* ence Bound';
label fiellcb = 'Fieller's*Conf.*Bounds';
label comment = ' ';
run;

proc print noobs split='*' data=output2 double;
by serotype pctile;
var igno igtype probt logldpct sd ldpct fiellcb deltabc;
format ldpct comma11.2;
title4 "PERCENTILES WITH CONFIDENCE INTERVALS BASED ON FIELLER'S AND DELTA METHODS";
run;

data jenndata.pctsep1; *data jenndata.pctsep1a;
merge output2 nout;
by serotype igno igtype;
keep serotype igtype igno pctile ldpct lcb ucb lcbd ucbd nanims slp halfwid;
halfwid = (ucb - lcb)/(2* ldpct) * 100;
run;

/* make output textfiles for table 1a and 1b to be pulled into Word */

data _null_;
length ig $15;
set jenndata.pctsep1;
*set jenndata.pctsep1a;
where igno not in (11,12);
if igtype='52-BIG' then ig='Task 97-52,BIG';
else if igtype='52-VIG' then ig='Task 97-52,VIG';
else ig=igtype;
file '\\pc-holdcraft\d\my projects\mref\task53\results\table1a.txt';
*file '\\pc-holdcraft\d\my projects\mref\task53\results\table1b.txt';
if _n_=1 then put "IG Type Used with Serotype A$N$Probit Slope(*)$LD50 (MIP LD50 Units per animal)$Fieller's 95 Percent Confid
put ig '$' nanims '$' slp :6.1 '$' ldpct :6.2 '$(' lcb :6.2 +(-1) ', ' ucb :6.2 +(-1) ')$'
halfwid :6.0;
run;

/* make notepad .onl files containing treatment group names, regression coefficients, and
covariance matrix using claire's format to be pulled into her program proratio.sas */

ata _null_;
set jenndata.estsep1;
where igno not in (11,12);
file '\\pc-holdcraft\d\my projects\mref\task53\results\proratio.onl';
put 'S Serotype_' serotype '1' igtype;
put slp int1;

```

```
put varb1;  
put covb0b1 varb0;  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
run;
```

```
data _null_;  
set jenndata.estsepta;  
where igno not in (11,12);  
file '\\pc-holdcraft\d\my projects\mref\task53\results\proratioa.onl';  
put 'S Serotype_' serotype '1 ' igtype;  
put slp int1;  
put varb1;  
put covb0b1 varb0;  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
put ' . . . . ';  
run;
```



```

/*****
**** Program Name: clinfish.sas
**** Purpose: runs fisher's exact tests on the incidence of signs
               for each serotype and dose level (in terms of GPdose),
               comparing incidences for BIG vs. VIG treated animals.
**** Project Number:
**** Date Written: 8/7/2000
**** Last Update: 11/30/2000
**** Data Sets Used: gpclin1.sd2
**** Programmer: Jennifer R. Holdcraft
*****/
libname sasdb '\\ns-bco-fs3\sasdata\mref\task53';
libname jenndata '\\pc-holdcraft\d\my projects\mref\task53\sas datasets';

```

```

options ls=120 ps=78;
title 'MREF Task 97-53 Clinical Signs Data';

```

```

proc format;
  value doselevl
    1 = '<= LD50' 2 = '> LD50';
  value yesno
    0 = 'Sign Absent' 1 = 'Sign Present';
run;

```

```

data d53;
  set sasdb.gpclin1 (keep=serotype date gpidd expnum igtype gpdose pcrit indth indsev indany);
  by gpidd notsorted;
  if first.gpidd;
  crit = substr(pcrit,1,3); drop pcrit;
  if igtype = 'PBIGA' then igno = 1;
  if igtype = 'PBIGB' then igno = 2;
  if igtype = 'PBIGAB' then igno = 3;
  if igtype = 'BBIG' then igno = 4;
  if igtype = 'CIG' then igno = 5;
run;

```

```

data d53a;
  set d53;
  if igno le 3 then do; igtype = '3PBIGS'; igno = 11; end;
  if igno = 5 then delete;
  compno = 1;
run;

```

```

data d53b;
  set d53;
  if igno le 4 then do; igtype = 'PBIGS+BBIG'; igno = 12; end;
  compno = 2;
run;

```

```

data gpclin;
  length igtype $10.;
  set d53a (in=in1) d53b (in=in2);
run;

```

```

* ld50's from probsep1.sas;
data lda;
  set jenndata.pctsep1 (keep=serotype ldpct igno igtype rename=(ldpct=ld50));
  if igno in (11,4);
  compno=1;
run;

```

```

data ldb;
  set jenndata.pctsep1 (keep=serotype ldpct igno igtype rename=(ldpct=ld50));
  if igno in (12,5);
  compno=2;
run;

```

```

data ld;
  set lda ldb;
  keep serotype igno igtype ld50;
  label ld50 = 'ld50';
run;

```

Program to perform Fisher's
 Exact Tests on incidence
 of signs for Table 1
 Jennifer Holdcraft
 12/5/2000

```

proc sort data=gpclin; by serotype igno igtype gpdose gpid; run;
proc sort data=ld; by serotype igno igtype; run;

* compare GP doses to individual group LD50s, then pool groups together;
data gpclin2;
  merge gpclin ld;
  by serotype igno igtype;

  if gpdose>ld50 then doselevl=2; else doselevl=1;
  if igtype = 'CIG' then igtype = 'ZCIG';

  if indth<30 then incid1=1; else incid1=0;
  if indsev<30 then incid2=1; else incid2=0;
  if indany<30 then incid3=1; else incid3=0;

  label incid1 = 'Death'
        incid2 = 'Severe signs (D/TP/WL)'
        incid3 = 'Any Sign';
run;

proc sort data=gpclin2; by serotype compno descending igno igtype doselevl; run;

title2 'LISTING OF INPUT DATA';
proc print data=gpclin2;
  by serotype compno descending igno igtype ld50;
  pageby compno;
  format ld50 10.2;
run;

proc sort data=gpclin2; by serotype compno doselevl descending igno igtype; run;

proc means noprint data=gpclin2;
  by serotype compno doselevl descending igno igtype;
  var gpdose;
  output out=nout (drop=_type_ _freq_) n=nanimals;
run;

title2 'Nos. of animals in each group';
proc print data=nout;
  by serotype compno doselevl notsorted;
run;

*****;

* Fisher's exact test results for endpoints variable ;

%macro dofreq(compno, endpt, igno1, igno2);

proc freq data=gpclin2;
  by serotype doselevl;
  where igno in (&igno1, &igno2);
  tables igtype* &endpt/exact nocol nopercnt out=freq&compno outpct;
  format doselevl doselevl. &endpt yesno.;
  output out=pval&compno exact;
run;

data freq&compno;
  set freq&compno;
  compno = &compno;
run;

data pval&compno;
  set pval&compno;
  compno = &compno;
run;

*
  oc print data=freq&compno;
  by serotype doselevl;
  format &endpt;
  title3 "freq&compno";

```

```

proc print data=pval&compno;
  by serotype doselevl;
  title3 "pval&compno";
run;
*/

%mend dofreq;

options pageno=1;
title 'Study No. G1555-53A      MREF Task 97-53 Clinical Signs Data';
title2 'Severe signs - Compare PBIGs to BBIG';
%dofreq(1, INCID2, 4, 11);

title2 'Severe signs - Compare PBIGs+BBIG to CIG';
%dofreq(2, INCID2, 5, 12);

title2 'Any signs - Compare PBIGs to BBIG';
%dofreq(3, INCID3, 4, 11);

title2 'Any signs - Compare PBIGs+BBIG to CIG';
%dofreq(4, INCID3, 5, 12);

*****;
data freqall;
  set freq1 freq2 freq3 freq4;
  format doselevl doselevl. incid2 incid3 yesno.;
run;

title3 'FREQALL';
proc print data=freqall;
  by serotype compno;
run;

data pvalues;
  set pval1 pval2 pval3 pval4;
  format doselevl doselevl. incid2 incid3 yesno.;
  in;

title3 'PVALUES';
proc print data=pvalues double noobs;
  by serotype; * compno;
run;

```

```

/*****
**** Program Name: clinsurv.SAS
**** Purpose: *gets data from gpclin dataset so can make a table of
the number of survivors by serotype and route and the number
of these animals that showed any sign other than normal in the
last week or on the last day
*frequencies of signs during last day and last week for survivors only;
**** Project Number:
**** Date Written: 3/16/98
**** Last Update: 11/30/2000
**** Data Sets Used: gpclin1.sd2
**** Data Sets Created:
**** Files Used:
**** Files Created:
**** Comments: creates table 2 for the Task 53 clinical observation report
**** Programmer: Jennifer A. Holdcraft
*****/
libname sasdb '\\ns-bco-fs3\sdasdata\mref\task53';

```

```

title 'MREF Task 97-53 Clinical Signs Data';

```

```

*proc contents data=sasdb.gpclin1;
*run;

```

```

proc sort data=sasdb.gpclin1 out=temp; by serotype igtype gpid; run;

```

```

* this data step creates the variables lastday and lastweek which will have values of
1 when the animal showed sign other than normal, and 0 when normal in the last week
and on last day of the study respectively ;

```

```

data clinsurv (keep=serotype date expnum igtype gpid lastday lastweek
tplstwk tplstdy);

```

```

set temp;
if indth=30; * index for time to death when death did not occur (survivors);
by serotype igtype gpid;
retain lastweek 0 lastday 0 tplstwk 0 tplstdy 0;
sumlstwk=sum (OF s15-s29);
sumlstdy=sum (OF s27-s29);
if first.gpid then do;
    if sumlstwk<15 then lastweek=1;
    else lastweek=0;
    if sumlstdy<3 then lastday=1;
    else lastday=0;
    tplstwk=0;
    tplstdy=0;
end;

```

```

* total paralysis or weak limbs;
if signno in (5,9) then do;
    if sumlstwk>0 then tplstwk=1;
    if sumlstdy>0 then tplstdy=1;
end;

```

```

if last.gpid then do;
    output;
end;
run;

```

```

proc sort data=clinsurv; by serotype igtype; run;

```

```

* creates dataset with then number of survivors for each serotype and route, and the
number of these animals that showed signs other than normal in the last week, or on
the last day of the study ;

```

```

options ls=120 ps=78;
proc means data=clinsurv n sum;
by serotype igtype;
var lastweek lastday tplstwk tplstdy;
output out=survstat
n=n
sum=weeksum daysum tpwksum tpdysum;
title2 'incidence of signs during last week and day';
run;

```

```

options ls=120 ps=62;

```

JAS program to tabulate
totals for survivors
shown in Table 2

Jennifer Holdcraft
12/5/2000

```
proc print data=survstat noobs double;  
var serotype igtype n weeksum daysum tpwksum tpdysum;  
run;
```

```
output to dollar delimited file so can make a table;  
data _null_;  
set survstat;  
file '\\pc-holdcraft\d\my projects\mref\task53\results\clinobs_tab2.txt';  
put serotype '$' igtype $char8. '$' (n weeksum daysum tpwksum tpdysum) (2.0 '$');  
run;
```

```

/*****
**** Program Name: lifettd.sas
**** Purpose: lifereg anova on time to death vs gp dose
**** Project Number:
**** Date Written:
**** Last Update: 11/30/2000
**** Data Sets Used:
**** Data Sets Created:
**** Files Used:
**** Files Created:
**** Comments: creates tables 3, 4, and 5 for the Task 53 clin
        obs report.
**** Programmer: Claire Matthews, Jennifer Holdcraft
*****/
libname sasdb '\\ns-bco-fs3\sdasdata\mref\task53';
libname jenndata '\\pc-holdcraft\d\my projects\mref\task53\sas datasets';

```

SAS program to perform
LIFEREG regression on time
to death + time to onset

of signs

Jennifer Holdcraft
12/5/2000

```
options ls=168 ps=59;
```

```
%let indvar = indth;
%let dropvars = indsev indany;
%let ttd = ttd;
%let signname = time to death;
run; quit;
```

```
/*
%let indvar = indsev;
%let dropvars = indth indany;
%let ttd = tsev;
%let signname = time to onset of severe signs;
run; quit;
```

```
/*
%let indvar = indany;
%let dropvars = indth indsev;
%let ttd = tany;
let signname = time to onset of any signs;
run; quit;
*/
```

```
* Create two datasets - the first one (comparison no.=1) compares the 3 combined
PBIG's versus BBIG. The second one (comparison no.=2) compare the 4 combined
PBIG's+BBIG versus CIG;
```

```
title1 'Study No. G1555-53A GP CLINICAL SIGNS';
title2 'MODEL OF &SIGNNAME - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS';
data d1a;
set sasdb.gpclin1 (drop= s1-s29 ld50ml1 ld50ml2 gpdose2 run pcr1t signno clinsign &dropvars dosedil);
by gpid notsorted;
if first.gpid;
```

```
ipred=0;
if &indvar=30 then do;
cens=1; &indvar=29;
end;
```

```
* convert index of checked box to half-day;
timonset = (&indvar-1)/2;
timonst1 = timonset;
timonst2 = timonset;
ltimons1 = log10(timonset);
ltimons2 = log10(timonset);
if cens=1 then do; timonst2=.; ltimons2 = .; end;
logdose = log10(gpdose);
```

```
if igtype in ('PBIGA', 'PBIGB', 'PBIGAB') then do; igtype = '3PBIGS'; igno = 11; end;
else if igtype = 'BBIG' then igno = 4;
else if igtype = 'CIG' then igno = 5;
compno = 1;
```

```
run;
```

```
data d1b;
```

```

set d1a;
if igno in (11,4) then do; igtype = 'PBIGS+BBIG'; igno = 12; end;
compno = 2;
run;

combine observed data from the two datasets together;
data d1;
set d1a(in=in1) d1b;
if in1 and igno = 5 then delete;
run;

proc sort data=d1; by compno igtype; run;

* for predicted lines;
data d2;
set d1 (keep=serotype igtype compno igno);
by compno igtype;
if first.igtype;
ipred=1;
min= 0; max= 6;
do logdose = min to max by .05;
gpdose = 10**logdose;
output;
end;
run;

* for specific predictions - GP dose LD50'S;
data lda;
set jennndata.pctsep1 (keep=serotype ldpct igno igtype rename=(ldpct=gpdose));
ipred=2;
logdose = log10(gpdose);
if igno in (11,4);
compno=1;
run;

data ldb;
set jennndata.pctsep1 (keep=serotype ldpct igno igtype rename=(ldpct=gpdose));
ipred=2;
logdose = log10(gpdose);
if igno in (12,5);
compno=2;
run;

* combine observed data, grid for predicted lines, and predicted values
at the LD50s together;

data d4;
set d1 d2 (drop=min max) lda ldb;
run;

proc sort data=d4; by serotype compno descending igno igtype ipred cens gpdose; run;

options ls=116 ps=78 pageno=1;
title3 'RIGHT-CENSORED REGRESSION OF LOG-GP &SIGNNAME VS LOG10(GP DOSE)';
proc lifereg data=d4 outest=outest;
by serotype compno descending igno igtype;
model (ltime1, ltime2) = logdose / dist=normal nolog ;
output out=outlif p=logpred std=stderr q= .25 .5 .75 ;
run; quit;

/*
filename newout 'd:\task53\lnlike.txt';
data _null_; set outest;
file newout;
put serotype igtype _lnlike_ 8.4;
run;
*/

data d5;
set outlif;
if ipred in (0,1) and _prob_ in (.25, .75) then delete;
if ipred=0 and cens=1 then ipred = 0.5;

```

```

if ipred ge 1 then timonset = 10**(logpred);
if timonset > 0 then lgtime = log10(timonset);

if ipred=1 then plotsym='.';
else if ipred le .5 then do;
  if cens le 0 then plotsym = 'd';
  if cens=1 then plotsym = 'l';
end;
else if ipred = 2 then plotsym = 'x';
run;

options ls=116 ps=78;
title3 'PREDICTED VALUES AT THE LD50 DOSES';
proc print data=d5;
  by serotype igtype logdose gpdose notsorted;
  id serotype igtype logdose gpdose;
  where ipred=2;
  var _prob_ lgtime stderr timonset;
  * var logdose dosedil _prob_ lgtime stderr timonset;
format gpdose best6. logdose lgtime 7.3 timonset stderr 6.3 gpdose 7.3;
run;

data jennndata.life&ttd;
  set d5;
  drop date timonst1 timonst2 plotsym ltimons1 ltimons2;
run;

*****;

* do t-test to compare predicted ttd at ld50s for BIG vs VIG;
data dtest;
  set jennndata.life&ttd;
  by serotype compno descending igno; *igtype;
  if ipred=2 and _prob_ = .5;
run;

title3 'DTEST - PREDICTED VALUES AT THE LD50 GPDOSSES';
proc print double noobs data=dtest;
run;

data dtest2;
  set dtest;
  by serotype compno;
  retain lpred1 lpred2 serr1 serr2 predtim1 predtim2;
  if first.compno then do; lpred1=.; lpred2=.; serr1=.; serr2=.; end;
  if igno in (11,12) then do; lpred1=logpred; serr1=stderr; predtim1=10**lpred1; end;
  if igno in (4,5) then do; lpred2=logpred; serr2=stderr; predtim2=10**lpred2; end;
  serrcomb = sqrt(serr1**2 + serr2**2);
  diff = (lpred1 - lpred2);
  zscore = diff / serrcomb;
  if last.compno;
  prob = probnorm(-1*abs(zscore)) * 2;
run;

options ls=116 ps=78;
title3 'COMPARE &SIGNNAME FOR 1ST VS 2ND GROUPS';
proc print double noobs;
  by serotype;
  var compno lpred1 serr1 predtim1 lpred2 serr2 predtim2 diff serrcomb zscore prob;
run;

*****;

data dpred;
  set jennndata.life&ttd (keep=serotype compno igno igtype gpdose _prob_ ipred timonset);
  by serotype compno descending igno; * igtype;
  if ipred=2;
  n;

* get predicted times and interquartile range at the LD50 condensed onto one record
  per serotype;

```



```

data dpred2;
  set dpred;
  by serotype compno descending igno;
  retain predtime q25 q75;
  if first.igno then do; predtime=.; q25=.; q75=.; end;
  if _prob_ = .25 then q25 = timonset;
  if _prob_ = .50 then predtime = timonset;
  if _prob_ = .75 then q75 = timonset;
  if last.igno;
  ld50 = gpdose;
  keep serotype compno igno igtype ld50 predtime q25 q75;
run;

*calculate nos. showing signs and GP doses for that subset;
data d6;
  set d4;
  if ipred = 0;
  n=1; nsign=1;
  if cens=1 then do; nsign=0; timonset = .; gpdose=.; end;
  drop timonst1 timonst2 ltimons1 ltimons2;
run;

/*
proc sort data=d6; by serotype igtype gpid; run;

options ls=120 ps=78 pageno=1;
title3 'DIAGNOSTIC LISTING SHOWING CALCULATED TIME TO ONSET';
proc print noobs data=d6; *(obs=50);
  by serotype igtype;
  format cens &indvar 3.0 timonset 5.1 serotype $char3.;
run;
*/

proc means noprint data=d6;
  by serotype compno descending igno;
  var timonset gpdose n nsign;
  output out=out1 (drop=_type_ _freq_)
  mean=avg&ttd avgdose std=std&ttd stderr=serr&ttd sum=dum1 dum2 ntot nsign;
run;

data d7;
  merge out1 (drop=dum1 dum2) dpred2;
  by serotype compno descending igno;
  if nsign=1 and serr&ttd=. then serr&ttd=0;
run;

options ls=116 ps=78 pageno=1 missing='-';
title3 'DESCRIPTIVE STATISTICS FOR &SIGNNAME - ANIMALS THAT SHOWED SIGN ONLY';
proc print data=d7 double;
  by serotype compno; id serotype compno;
  var igno igtype ntot nsign avgdose avg&ttd std&ttd ld50 predtime q25 q75;
  format avg&ttd predtime q25 q75 7.3 std&ttd serr&ttd 6.3;
  format serotype $char2.;
run;
options missing='-';
run;

data _null_;
  set d7;
  *file "\\pc-holdcraft\d\my projects\mref\task53\results\clinobs_tab3.txt";
  *file "\\pc-holdcraft\d\my projects\mref\task53\results\clinobs_tab4.txt";
  file "\\pc-holdcraft\d\my projects\mref\task53\results\clinobs_tab5.txt";
  length desc&ttd $12 bounds $20;
  desc&ttd = put(avg&ttd,4.1) || ' (' || put(std&ttd,3.1) || ')';
  if nsign le 0 then desc&ttd = ' -';

  bounds = put(q25,3.1) || ' - ' || put(q75,4.1);

  put serotype $char2. '$'
  igtype $char10. '$'
  ntot 3.0 '$'
  nsign 3.0 '$'
  avgdose 6.1 '$'

```

**** Program Name: corrtime.sas

**** Purpose: gets correlations quoted in text of Task 53 clinical observation report.

**** Project Number:

**** Date Written:

**** Last Update: 12/01/2000

**** Data Sets Used:

**** Data Sets Created:

**** Files Used:

**** Files Created:

**** Comments:

**** Programmer: Claire Matthews, Jennifer Holdcraft

libname sasdb '\\ns-bco-fs3\sasdata\mref\task53';

libname jenndata '\\pc-holdcraft\d\my projects\mref\task53\sas datasets';

options ls=100 ps=78;

* read duration dataset created by genmodrn.sas;

data d1;

set jenndata.duration (drop=a1-a29 s1-s29);

label indany = 'Time to onset of any sign';

label indsev = 'Time to onset of severe signs';

label indth = 'Time to death';

label durany = 'Duration of any sign';

label dursev = 'Duration of severe signs';

run;

proc sort data=d1; by igtype serotype gp1d; run;

proc corr data=d1;

*by igtype;

*by serotype;

var indth indany indsev;

*where indth < 30 or indany < 30 or indsev < 30;

in;

title 'TASK 53 TIME TO ONSET OF CLINICAL SIGNS';

title2 'CORRELATIONS AMONG TIMES TO ONSET FOR ALL ANIMALS - IG TYPES COMBINED';

proc corr data=d1;

*by igtype;

var durany dursev;

title 'TASK 53 DURATIONS OF CLINICAL SIGNS';

title2 'CORRELATIONS AMONG DURATIONS OF SIGNS (NUMBER OF BOXES CHECKED OUT OF 28)';

run;

options ls=170 ps=59;

proc plot data=d1;

plot indsev*indany dursev*durany/haxis=0 to 30 by 5 vaxis=0 to 30 by 5;

title 'TASK 53 CLINICAL SIGNS';

title2 'plots showing correlations between onset and duration';

run;

quit;

Program to compute Pearson's
correlations of durations of
severe signs + any signs

Jennifer Holdcraft
12/5/2000

```

/*****
**** Program Name: genmodrn.sas
**** Purpose: probit regression on durations of signs
**** Project Number:
**** Date Written:
**** Last Update: 12/01/2000
**** Data Sets Used:
**** Data Sets Created:
**** Files Used:
**** Files Created:
**** Comments: creates Table 6 in Task 53 clinical observation report
**** Programmer: Claire Matthews, Jennifer Holdcraft
*****/
libname sasdb '\\ns-bco-fs3\sdasdata\mref\task53';
libname jenndata '\\pc-holdcraft\d\my projects\mref\task53\sas datasets';

options ls=120 ps=78;

/*
* first create a SAS dataset with durations (numbers of boxes checked);
data d1;
  set sasdb.gpolin1;
  if signno > 1;
run;

proc sort data=d1; by serotype igtype gpid; run;

* any sign;
proc means data=d1 noprint;
  by serotype igtype gpid;
  var s1-s29;
  id gpdose expnum date indth indany indsev;
  output out=out1 (drop=_type_ _freq_)
    sum=suma1-suma29;
run;

  severe signs (weak limbs, total paralysis, death);
proc means data=d1 noprint;
  where signno in (5,9,11);
  by serotype igtype gpid;
  var s1-s29;
  id indth;
  output out=out2 (drop=_type_ _freq_)
    sum=sumb1-sumb29;
run;

data d2;
  merge out1 out2;
  by serotype igtype gpid;
  array sumas{29} suma1-suma29;
  array sumbs{29} sumb1-sumb29;

  durany = 0; dursev = 0;
  do i=2 to 29;
    if sumas{i} > 1 then sumas{i} = 1;
    if sumbs{i} > 1 then sumbs{i} = 1;
    durany = sum(sumas{i}, durany);
    dursev = sum(sumbs{i}, dursev);
  end;

  drop i suma1-suma29 sumb1-sumb29;

* If GP died before end of observation period, pad out interval length
to the end (duration of time dead is also included in duration of signs);

  if indth < 29 then do;
    durany = durany + (29-indth);
    dursev = dursev + (29-indth);
  end;
run;

* get indicator variables for each time slot for whether any sign occurred.

```

*SAS program to perform
modeling on durations
of any signs*

*Jennifer Holdcraft
12/5/2000*

```

    If animal died, pad out the remaining indicator vars with ones;

data out1a;
    set out1;
    array sumas{29} suma1-suma29;
    array anys{29} a1-a29;
    do i=2 to 29;
        if      sumas{i} = . then anys{i}=0;
        else if sumas{i} ge 1 then anys{i}=1;
        else if sumas{i} eq 0 then anys{i}=0;
    end;

    if indth < 29 then do;
        do i=indth to 29;
            anys{i} = 1;
        end;
    end;
    keep serotype igtype gpid a1-a29;
run;

data out2a;
    set out2;
    array sumbs{29} sumb1-sumb29;
    array sevs{29} s1-s29;
    do i=2 to 29;
        if      sumbs{i} = . then sevs{i}=0;
        else if sumbs{i} ge 1 then sevs{i}=1;
        else if sumbs{i} eq 0 then sevs{i}=0;
    end;

    if indth < 29 then do;
        do i=indth to 29;
            sevs{i} = 1;
        end;
    end;
    keep serotype igtype gpid s1-s29;
run;

data jenndata.duration;
    merge d2 out1a out2a;
    by serotype igtype gpid;
run;

proc sort data=sasdb.duration; by serotype igtype gpdose; run;

options ls=174 ps=61 pageno=1;
title 'TASK 53 CLINICAL SIGNS';
title2 'DURATION.SD2 - INDICATOR VARS FOR OCCURRENCE OF ANY SIGN AND SEVERE SIGNS';
proc print data=jenndata.duration;
    by serotype igtype;
    id serotype igtype;
    var gpid expnum gpdose indth--dursev a2-a29 s2-s29;
    format expnum 6.0 gpdose 6.2 indth durany a2 s2 4.0;
run;
*/

*****;
%let yvar = durany;
%let yname = proportion of time showing any sign;
%let var29s = a1-a29;
/*
%let yvar = dursev;
%let yname = proportion of time showing severe signs;
%let var29s = s1-s29;
*/
run;

data dinput;
    set jenndata.duration (drop=indth indany indsev);
    ipred=0;
    plotsym = substr(igtype,2,1);
    logdose = log10(gpdose);

```

```

if igtype in ('PBIGA', 'PBIGB', 'PBIGAB') then do; igtype = '3PBIGS'; igno = 11; end;
else if igtype = 'BBIG' then igno = 4;
else if igtype = 'CIG' then igno = 5;
compno = 1;
run;

```

```

data da;
set dinput;
if igno in (11,4) then do; igtype = 'PBIGS+BBIG'; igno = 12; end;
compno = 2;
run;

```

* combine observed data from the two datasets together;

```

data d2;
set dinput(in=in1) da;
if in1 and igno = 5 then delete;
run;

```

* for specific predictions - GP dose LD50'S;

```

data lda;
set jennndata.pctsep1 (keep=serotype ldpct igno igtype rename=(ldpct=gpdose));
ipred=1;
logdose = log10(gpdose);
if igno in (11,4);
compno=1;
run;

```

```

data ldb;
set jennndata.pctsep1 (keep=serotype ldpct igno igtype rename=(ldpct=gpdose));
ipred=1;
logdose = log10(gpdose);
if igno in (12,5);
compno=2;
run;

```

```

data ldab;
set lda ldb;
nn = _n_;
run;

```

* combine observed data, grid for predicted lines, and predicted values at the LD50s together;

```

data d3;
set d2 ldab;
label logdose = 'LOG10 OF GP DOSE (MIPLD50 UNITS/ANIMAL)';
*label durany = 'Prop. of observed time with any sign';
*label dursev = 'Prop. of observed time with severe sign';
run;

```

* for predicted lines;

```

data d4;
length igtype $10;
ipred=2;
serotype = 'A';

do igno = 4, 5, 11, 12;
if igno in (4,11) then compno = 1;
if igno in (5,12) then compno = 2;
if igno = 11 then igtype = '3PBIGS';
if igno = 12 then igtype = 'PBIGS+BBIG';
if igno = 4 then igtype = 'BBIG';
if igno = 5 then igtype = 'CIG';

```

```

min=0; max= 6;
do logdose = min to max by .1;
gpdose = 10**logdose;
output;
end;
end;
run;

```

```

data d4;
  set d4;
  nn = _n_;
run;

data dall;
  set d3 d4 (drop=min max);
  * if ipred=0 then ninterv = 28;
  * else ninterv=1;
  drop s1-s29;
run;

proc sort data=dall; by serotype compno descending igno igtype ipred gpdose; run;

data dall29;
  set dall; * (drop=ninterv);
  drop i a1-a29 nn;
  nint = 1;
  array signs{29} &var29s;

  if ipred=0 then do;
    do i=2 to 29;
      seq = i;
      indsign = signs{i};
      output;
    end;
  end;

  * get unique, new gp id to each of hypothetical records;
  else do;
    seq=2;
    if ipred=1 then gpid = put(1000 + nn, 4.0);
    if ipred=2 then gpid = put(2000 + nn, 4.0);
    output;
  end;
in;

data dall29;
  set dall29;
  if ipred le 1;
run;

proc sort data=dall29; by serotype compno descending igno igtype ipred; run;

*proc printto new print='d:\task53\genmodrn.lst';

options ls=120 ps=78 pageno=1;
ods listing close;
ods output obstats=genout;
proc genmod data=dall29 order=data;
  by serotype compno descending igno igtype;
  *where ipred le 1;
  class gpid;
  model indsign/nint = logdose / obstats dist=bin link=probit;
  repeated subject=gpid / type=exch; * corrw;
  *make 'geeobstats' out=genout;
  title 'TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYP E';
  title2 'PROC GENMOD ON &YNAME VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT';
run; quit;
ods listing;

*proc printto; *run;

/*
data _null_;
  set outest;
  file 'd:\task53\lnlike.txt';
  put serotype igtype _lnlike_ 8.4;
run;
*/

```

```

data d6;
  merge dall29 genout (keep=pred xbeta std lower upper rename={std=stdxbeta pred=predprp});
  drop seq nint;
  if ipred=0 then do;
    plotsym = substr(igtype,2,1);
    prpdurn = &yvar/28;
  end;
  else do;
    prpdurn = predprp;
    if ipred=1 then plotsym = 'x';
    if ipred=2 then plotsym = '.';
  end;
  label prpdurn = "Proportion Time for &yvar";
run;

/*
options ls=170 ps=59 pageno=1;
proc plot nolegend data=d6;
  by serotype igtype;
  plot prpdurn*logdose = plotsym / vaxis = 0 to 1 by .1;
title3 'PLOT OF OBSERVED AND PREDICTED VALUES';
run;
*/

data jenndata.geprdurn;
  set d6 (drop=plotsym);
run;

data d7;
  set jenndata.geprdurn;
  if ipred=1;
  preddurn = predprp*14;
  lodurn = lower*14;
  updurn = upper*14;
  drop date durany dursev indsign gpid expnum prpdurn;
run;

options ls=168 ps=59;
proc print data=d7;
  by serotype; * igtype ipred;
  id serotype; * igtype ipred;
  format predprp xbeta 7.4 logdose 6.3 gpdose 6.2;
  title 'TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYPE';
  title2 "PROC GENMOD ON &YNAME VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT";
  title3 'PREDICTED VALUES AT THE LD50 DOSES';
run;

data dpred;
  set d7 (keep=serotype igtype igno compno ipred gpdose predprp lower upper
    preddurn lodurn updurn gpdose rename={gpdose=ld50});
run;

data dobs;
  set jenndata.duration (keep=serotype igtype gpid gpdose durany);
  if igtype in ('PBIGA', 'PBIGB', 'PBIGAB') Then do;
    igtype = '3PBIGS';
    igno = 11;
  end;
  else if igtype = 'BBIG' then igno = 4;
  else if igtype = 'CIG' then igno = 5;
  compno = 1;
run;

data dobsa;
  set dobs;
  if igno in (11,4) then do; igtype = 'PBIGS+BBIG'; igno = 12; end;
  compno = 2;
run;

* combine observed data from the two datasets together;
data dobs2;
  set dobs(in=in1) dobsa;
  if in1 and igno = 5 then delete;

```

```

ntot=1;
nsign=1;
durany = durany/2; *convert numbers of boxes observed to days;
if durany=0 then do; nsign=0; gpdose=.; prpdurn=.; durany=.; end;
ipred = 0;
in;

proc sort data=dobs2; by serotype compno descending igno igtype ipred gpdose; run;

proc means noprint data=dobs2;
  by serotype compno descending igno igtype;
  var gpdose durany ntot nsign;
  output out=nout (drop=_type_ _freq_ dum1 dum2)
    mean=avgdose avgdurn
    std=stddose stddurn
    sum=dum1 dum2 ntot nsign;
run;

title2 'DESCRIPTIVE STATS FOR ANIMALS THAT SHOWED SIGNS';
proc print data=nout;
  by serotype compno;
  id serotype compno;
  var igtype ntot nsign avgdose stddose avgdurn stddurn;
run;

*****;

* do t-test to compare predicted ttd at ld50s for 2 pairs of IG Types;
data dtest;
  set jennndata.geprdurn;
  by serotype compno;
  if ipred=1;
run;

data dtest2;
  set dtest;
  by serotype compno;

  retain ld501 ld502 xbeta1 xbeta2 serr1 serr2 pred1 pred2;
  length pred1 pred2 $30;
  if first.compno then do;
    ld501=.; ld502=.; xbeta1=.; xbeta2=.; serr1=.; serr2=.;
    pred1=' '; pred2=' ';
  end;

  if first.compno then do;
    ld501=gpdose; xbeta1=xbeta; serr1=stdxbeta;
    pred1 = put(prpdurn,6.4) || ' (' || put(lower,6.4) || ', ' || put(upper,6.4) || ')';

    * predval = probnorm(xbeta);
    * lcb = probnorm(xbeta - 1.96*stdxbeta);
    * ucb = probnorm(xbeta + 1.96*stdxbeta);
    * pred1 = put(predval,6.4) || ' (' || put(lcb,6.4) || ', ' || put(ucb,6.4) || ')';
  end;

  else if last.compno then do;
    ld502=gpdose; xbeta2=xbeta; serr2=stdxbeta;
    pred2 = put(prpdurn,6.4) || ' (' || put(lower,6.4) || ', ' || put(upper,6.4) || ')';

    * predval = probnorm(xbeta);
    * lcb = probnorm(xbeta - 1.96*stdxbeta);
    * ucb = probnorm(xbeta + 1.96*stdxbeta);
    * pred2 = put(predval,6.4) || ' (' || put(lcb,6.4) || ', ' || put(ucb,6.4) || ')';
  end;

  * drop predval lcb ucb;

  serrcomb = sqrt(serr1**2 + serr2**2);
  diff = (xbeta1 - xbeta2);
  zscore = diff / serrcomb;
  prob = (1-probnorm(abs(zscore))) * 2;
  if last.compno;
run;

```



```

*OPTIONS LS=168 PS=59;
TITLE2 "DO NORMAL Z-SCORES TO COMPARE &YNAME FOR 1ST AND 2ND IG TYPE(S)";
proc print double noobs;
  by serotype;
  var compno ld501 xbeta1 serr1 pred1
      ld502 xbeta2 serr2 pred2 diff serrcomb zscore prob;
  format ld501 ld502 6.2 xbeta1 xbeta2 7.4 serrcomb 7.5 zscore 6.3 prob 7.5 ;
run;

```

```

data d8;
  merge dpred nout;
  length descurn $12 bounds $20;
  descurn = put(avgdurn,4.1) || ' (' || put(stddurn,3.1) || ')';
* bounds = put(lower,3.1) || ' - ' || put(upper,3.1);
  bounds = put(lodurn,4.1) || ' - ' || put(updurn,4.1);
run;

```

```

title2 'TABLE 6';
proc print data=d8 double noobs;
  by serotype;
  var igtype ntot nsign avgdose descurn ld50 preddurn bounds;
run;

```

```

*****;

```

```

data _null_;
  set d8;
  file "\\pc-holdcraft\d\my projects\mref\task53\results\clinobs_tab6.txt";
  put serotype '$'
  igtype $char10. '$'
  ntot 3.0 '$'
  nsign 3.0 '$'
  avgdose 6.1 '$'
  descurn $char10. '$'
  ld50 6.2 '$'
  preddurn 5.1 '$'
  bounds $char12.;
run;

```

/*****

**** Program Name: freqcomm.sas

**** Purpose:

**** Project Number:

**** Date Written:

**** Last Update: 11/30/2000

**** Data Sets Used:

**** Data Sets Created:

**** Files Used:

**** Files Created:

**** Comments: creates Table 7 in Task 53 clinical observation report

**** Programmer: Claire Matthews, Jennifer Holdcraft

*****/

libname sasdb '\\ns-bco-fs3\sasdata\mref\task53';

libname jenndata '\\pc-holdcraft\d\my projects\mref\task53\sas datasets';

options ls=110 ps=78;

data clin;

set sasdb.gpolin1 (keep=serotype gpid igtype signno clinsign s1-s29);

indsign = sum (of s1-s29);

if indsign ge 1 then indsign = 1; else if indsign ne . then indsign = 0;

drop s1-s29;

run;

proc sort data=clin; by igtype signno gpid; run;

title 'TASK 53 CLINICAL SIGNS - TOTAL NOS. OF ANIMALS PER GROUP';

proc means data=clin noprint;

by serotype igtype signno clinsign;

where signno > 1;

var indsign;

output out=nout (drop=_type_ _freq_) sum=nsign n=total;

run;

data dtot;

set nout (keep=serotype igtype total);

by serotype igtype;

if first.igtype;

run;

*proc print data=nout;

*by igtype;

*run;

data clin2;

set nout;

percent = nsign/total * 100;

outpct = put(nsign,3.0) || ' (' || put(percent,2.0) || ')';

run;

proc print data=clin2;

by igtype total;

format percent 6.1;

run;

proc sort data=clin2; by serotype signno igtype; run;

proc transpose data=clin2 out=tran;

by serotype signno clinsign;

var outpct;

id igtype;

run;

data clin3;

set tran (drop=_name_);

*serotype = 'A';

in;

proc print data=clin3;

by serotype;

run;

Program to tabulate incidence of
each type of sign for Table 7

Jennifer R Holdcraft
12/5/2000

*****;

```
data comm;
  set sasdb.clincomm (keep=serotype gpid igtype comment);
  comment = upcase(comment);
  format comment;
run;
```

```
proc sort data=comm; by igtype gpid; run;
```

```
/*
title 'TASK 53 CLINICAL SIGNS - OTHER SYMPTOMS';
title2 'ITEMIZE ALL SYMPTOMS LISTED IN THE COMMENT FIELD INCLUDING ALL SPELLINGS';
options ls=106 ps=78 pageno=1;
proc freq data=comm;
  tables comment;
  where comment ne ' ';
run;
*/
```

```
* cluster signs together into logical group to take into account
  various spellings and wordings of comments;
```

```
data chk;
  length comment $45;
  set comm;
  if comment ne ' ';
  if comment in ('ANIMAL EUTHANIZED DUE TO MORIBUND CONDITION' ,
    'ANIMAL EUTHANIZED DUE TO MORIBUND CONDITIONS' ,
    'EUTHANIZED DUE TO MORIBUND CONDITION') then do;
    comment = 'Moribund/Euthanized'; output; end;

  else if comment in ('ANIMAL HAD PROLAPSED RECTUM AT DEATH',
    'PROLAPSED RECTUM',
    'PROLAPSED PENIS') then do;
    comment = 'Prolapsed rectum/Prolapsed penis'; output; end;

  else if comment in ('DEHYDRATED',
    'DIARRHEA',
    'HAIR LOSS') then do; output; end;

  else if comment in ('BLOODY DIARRHEA', 'BLEEDING FROM HIND END') then do;
    comment='Bloody diarrhea/Bleeding from hind end'; output; end;

  else if comment in ( 'DEHYDRATED AND DIARRHEA',
    'DEHYDRATION AND DIARRHEA',
    'DIARRHEA AND DEHYDRATED',
    'DIARRHEA/DEHYDRATED') then do;
    comment = 'Diarrhea'; output;
    comment = 'Dehydrated'; output; end;

  else if comment = 'DEHYDRATED AND HAIR LOSS' then do;
    comment = 'Dehydrated'; output;
    comment = 'Hair loss'; output; end;

  else if comment = 'DIARRHEA AND RUNNY NOSE' then do;
    comment = 'Diarrhea'; output;
    comment = 'Runny nose'; output; end;

  else do; output; end;
run;
```

```
data chk;
  set chk;
  comment = upcase(comment);
run;
```

```
roc sort data=chk; by gpid comment; run;
```

```
data chk2;
  set chk;
  by gpid comment;
  if last.gpid or last.comment;
```

```

one=1;
run;

proc sort data=chk2; by comment; run;

ita chk3;
set chk2;
by comment;
* note - sign nos. 1 through 11 were used to number the eight standard signs
when frequencies were previously computed by Crtclins.Sas;
retain signno;
if _n_ = 1 then signno = 12;
else if first.comment then signno = signno + 1;
run;

proc sort data=chk3; by serotype igtype comment; run;

proc means data=chk3 noprint;
by serotype igtype comment;
var one;
id signno;
output out=sumout (drop=_type_) sum=nsign;
run;

data sumout2;
merge sumout (rename=comment=clinsign) dtot;
by serotype igtype;
percent = (nsign/total) * 100;
outpct = put(nsign,3.0) || ' (' || put(percent,2.0) || ')';
run;

options ls=110 ps=78 pageno=1;
title1 ' ';
title2 'TASK 53 CLINICAL SIGNS - OTHER SYMPTOMS (GROUPED)';
title3 'FREQUENCIES AND PERCENTS OF ANIMALS SHOWING SIGNS AT LEAST ONCE';
proc print data=sumout2;
by serotype igtype total;
id serotype igtype;
var signno clinsign nsign percent outpct;
format percent 6.1;
run;

proc sort data=sumout2; by serotype signno igtype; run;

proc transpose data=sumout2 out=tran2;
by serotype signno clinsign;
var outpct;
id igtype;
run;

data comm3;
set tran2 (drop=_name_);
array grps{5}$ bbig cig pbiga pbigab pbigb;
do i=1 to 5;
if grps{i} = ' ' then grps{i} = '-';
end;
drop i;
run;

data comb;
length clinsign $40;
set clin3 comm3;
run;

proc print data=comb;
by serotype;
var signno clinsign pbiga pbigb pbigab bbig cig;
run;

data _null_;
set comb;
file '\\pc-holdcraft\d\my projects\mref\task53\results\clinobs_tab7.txt';
put signno 2.0 '$' clinsign '$'

```

```
(pbiga pbigh pbigab bbig cig) (char8. '$');  
run;
```

17:08 Tuesday, November 21, 200

SEROTYPE=A igno=11 IGTYPE=3PBIGS

Probit Procedure

Probit Analysis on GPDOSE

Probability	GPDOSE	95% Fiducial Limits	
0.01	8292	754.49257	19867
0.02	11228	1339	24508
0.03	13609	1927	28017
0.04	15728	2531	30998
0.05	17693	3160	33667
0.06	19557	3815	36128
0.07	21352	4499	38444
0.08	23099	5214	40652
0.09	24812	5962	42780
0.10	26501	6743	44845
0.15	34805	11193	54661
0.20	43224	16671	64264
0.25	52053	23340	74224
0.30	61508	31367	85035
0.35	71797	40895	97296
0.40	83147	51983	111868
0.45	95833	64546	130063
0.50	110206	78327	153830
0.55	126734	93023	185903
0.60	146070	108533	230023
0.65	169161	125120	291618
0.70	197457	143431	379476
0.75	233324	164538	509321
0.80	280983	190216	712405
0.85	348953	223783	1060304
0.90	458302	272904	1759352
0.91	489491	286107	1989610
0.92	525785	301105	2274557
0.93	568805	318427	2635842
0.94	621025	338864	3108409
0.95	686459	363670	3752742
0.96	772198	395007	4683987
0.97	892414	437069	6153893
0.98	1081678	499700	8850723
0.99	1464720	616479	15710627

Output from PROBSEP1.sas - result
is LD50 for pooled PBIG groups,
quoted in report "Methods" section.

Jennifer Holdcraft
12/5/2000

17:08 Tuesday, November 21, 200

SEROTYPE=A igno=12 IGTYP=PBIGS+BBIG

Probit Procedure

Probit Analysis on GPDOSE

Probability	GPDOSE	95% Fiducial Limits	
0.01	12354	2747	23989
0.02	16069	4237	29064
0.03	18987	5574	32846
0.04	21525	6850	36025
0.05	23838	8097	38848
0.06	26002	9334	41435
0.07	28061	10571	43854
0.08	30041	11814	46148
0.09	31964	13069	48348
0.10	33842	14339	50475
0.15	42868	21003	60462
0.20	51730	28336	70061
0.25	60778	36477	79853
0.30	70246	45517	90290
0.35	80330	55505	101863
0.40	91235	66427	115206
0.45	103192	78195	131169
0.50	116489	90686	150882
0.55	131499	103838	175792
0.60	148733	117748	207775
0.65	168923	132740	249462
0.70	193173	149391	304946
0.75	223263	168615	381204
0.80	262317	191929	491357
0.85	316541	222184	663564
0.90	400965	265944	972686
0.91	424528	277604	1067359
0.92	451697	290801	1180887
0.93	483583	305979	1319951
0.94	521862	323803	1495013
0.95	569231	345323	1723580
0.96	630406	372340	2037694
0.97	714695	408331	2504201
0.98	844440	461401	3295243
0.99	1098390	558928	5083381

Output from PROBSEP1.sas - result
is LD50 for pooled PBIG's and BBIG
groups - quoted in report "Methods"
section

Jennifer Holdcraft
12/5/2000

SEROTYPE=A

igno	igtype	lnlike	regtype	septot3	septot1	chisq	df	pvalue
1	PBIGA	-22.5831	1	-22.5831	.	.	.	.
2	PBIGB	-9.6364	1	-32.2195	.	.	.	.
3	PBIGAB	-21.2679	1	-53.4874	.	.	.	.
11	3PBIGS	-57.2876	2	-53.4874	-57.2876	7.60037	4	0.10736

This + next page - output from PROBSEP1.sas
loglikelihood tests. P-values are
not significant ($p < 0.05$) indicating
no loss of fit caused by pooling data,
quoted in text "Methods" section

Jennifer Holdcraft
12/5/2000

Study No. G1555-53A - use unadjusted GP doses 31
See if 3 PBIGs + BBIG can be combined into a single probit curve
11:53 Tuesday, December 5, 2000

----- SEROTYPE=A -----

igno	igtype	lnlike	regtype	septot3	septot1	chisq	df	pvalue
1	PBIGA	-22.5831	1	-22.5831	.	.	.	.
2	PBIGB	-9.6364	1	-32.2195	.	.	.	.
3	PBIGAB	-21.2679	1	-53.4874	.	.	.	.
4	BBIG	-10.0569	1	-63.5443	.	.	.	.
12	PBIGS+BBIG	-69.4670	2	-63.5443	-69.4670	11.8454	6	0.065507

SEROTYPE=A doselev1=<= LD50

The FREQ Procedure

Table of igtype by incid2

igtype(IGTYPE) incid2(Severe signs (D/TP/WL))

Frequency Row Pct	Sign Absent	Sign Present	Total
<u>3PBIGS</u>	14 23.73	45 <u>76.27</u>	59
<u>BBIG</u>	3 14.29	18 <u>85.71</u>	21
Total	17	63	80

Statistics for Table of igtype by incid2

Statistic	DF	Value	Prob
Chi-Square	1	0.8253	0.3636
Likelihood Ratio Chi-Square	1	0.8790	0.3485
Continuity Adj. Chi-Square	1	0.3574	0.5499
Mantel-Haenszel Chi-Square	1	0.8150	0.3667
Phi Coefficient		0.1016	
Contingency Coefficient		0.1010	
Cramer's V		0.1016	

WARNING: 25% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	14
Left-sided Pr <= F	0.8920
Right-sided Pr >= F	0.2823
Table Probability (P)	0.1743
Two-sided Pr <= P	<u>0.5369</u>

Sample Size = 80

Output from CLINFISH.sas for Table 1
 of Task 53 Statistical Analysis of
 Clinical Signs Data Report. Similar output follows.

Jennifer R Holdcraft
 12/1/2000

SEROTYPE=A doselevl=> LD50

The FREQ Procedure

Table of igtype by incid2

igtype(IGTYPE)		
incid2(Severe signs (D/TP/WL))		
Frequency Row Pct	Sign Present	Total
3PBIGS	51 100.00	51
BBIG	9 100.00	9
Total	60	60

Note: Fisher's Exact Test
 Cannot be performed when both
 groups have 100% present so
 p-value is represented as 1.000
 to indicate similarity of groups.

Jennifer R Holdcraft
 12/1/2000

SEROTYPE=A doselevl=<= LD50

The FREQ Procedure

Table of igtype by incid2

igtype(IGTYPE) incid2(Severe signs (D/TP/WL))

Frequency Row Pct	Sign Absent	Sign Present	Total
<u>PBIGS+BBIG</u>	17 21.25	63 <u>78.75</u>	80
<u>ZCIG</u>	2 6.90	27 <u>93.10</u>	29
Total	19	90	109

Statistics for Table of igtype by incid2

Statistic	DF	Value	Prob
Chi-Square	1	3.0467	0.0809
Likelihood Ratio Chi-Square	1	3.5440	0.0598
Continuity Adj. Chi-Square	1	2.1310	0.1443
Mantel-Haenszel Chi-Square	1	3.0188	0.0823
Phi Coefficient		0.1672	
Contingency Coefficient		0.1649	
Cramer's V		0.1672	

Fisher's Exact Test

Cell (1,1) Frequency (F)	17
Left-sided Pr <= F	0.9857
Right-sided Pr >= F	0.0659
Table Probability (P)	0.0516
Two-sided Pr <= P	<u>0.0939</u>

Sample Size = 109

SEROTYPE=A doselevl=> LD50

The FREQ Procedure

Table of igtype by incid2

igtype(IGTYPE)		incid2(Severe signs (D/TP/WL))	
Frequency			
Row Pct	Sign Present	Total	
PBIGS+BBIG	60	60	
	<u>100.00</u>		
ZCIG	11	11	
	<u>100.00</u>		
Total	71	71	

SEROTYPE=A doselevl=<= LD50

The FREQ Procedure

Table of igtype by incid3

igtype(IGTYPE) incid3(Any Sign)

Frequency Row Pct	Sign Absent	Sign Present	Total
3PBIGS	11 18.64	48 81.36	59
BBIG	2 9.52	19 90.48	21
Total	13	67	80

Statistics for Table of igtype by incid3

Statistic	DF	Value	Prob
Chi-Square	1	0.9466	0.3306
Likelihood Ratio Chi-Square	1	1.0377	0.3084
Continuity Adj. Chi-Square	1	0.3950	0.5297
Mantel-Haenszel Chi-Square	1	0.9347	0.3336
Phi Coefficient		0.1088	
Contingency Coefficient		0.1081	
Cramer's V		0.1088	

WARNING: 25% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	11
Left-sided Pr <= F	0.9126
Right-sided Pr >= F	0.2739
Table Probability (P)	0.1865
Two-sided Pr <= P	0.4962

Sample Size = 80

SEROTYPE=A doselevl=> LD50

The FREQ Procedure

Table of igtype by incid3

igtype(IGTYPE)		incid3(Any Sign)	
Frequency			
Row Pct	Sign Present	Total	
<u>3PBIGS</u>	51 <u>100.00</u>	51	
<u>BBIG</u>	9 <u>100.00</u>	9	
Total	60	60	

SEROTYPE=A doselevl=<= LD50

The FREQ Procedure

Table of igtype by incid3

igtype(IGTYPE)		incid3(Any Sign)	
Frequency Row Pct	Sign Absent	Sign Present	Total
PBIGS+BBIG	13 16.25	67 83.75	80
ZCIG	2 6.90	27 93.10	29
Total	15	94	109

Statistics for Table of igtype by incid3

Statistic	DF	Value	Prob
Chi-Square	1	1.5691	0.2103
Likelihood Ratio Chi-Square	1	1.7708	0.1833
Continuity Adj. Chi-Square	1	0.8799	0.3482
Mantel-Haenszel Chi-Square	1	1.5547	0.2124
Phi Coefficient		0.1200	
Contingency Coefficient		0.1191	
Cramer's V		0.1200	

WARNING: 25% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	13
Left-sided Pr <= F	0.9504
Right-sided Pr >= F	0.1756
Table Probability (P)	0.1260
Two-sided Pr <= P	0.3456

Sample Size = 109

SEROTYPE=A IGTYPE=BBIG

The MEANS Procedure

Variable	N	Sum
lastweek	20	18.0000000
lastday	20	16.0000000
tplstwk	20	15.0000000
tplstdy	20	9.0000000

SEROTYPE=A IGTYPE=CIG

Variable	N	Sum
lastweek	28	25.0000000
lastday	28	25.0000000
tplstwk	28	25.0000000
tplstdy	28	25.0000000

SEROTYPE=A IGTYPE=PBIGA

Variable	N	Sum
lastweek	21	18.0000000
lastday	21	18.0000000
tplstwk	21	12.0000000
tplstdy	21	9.0000000

SEROTYPE=A IGTYPE=PBIGAB

Variable	N	Sum
lastweek	20	16.0000000
lastday	20	16.0000000
tplstwk	20	14.0000000
tplstdy	20	11.0000000

SEROTYPE=A IGTYPE=PBIGB

Variable	N	Sum
lastweek	21	17.0000000
lastday	21	17.0000000
tplstwk	21	9.0000000
tplstdy	21	8.0000000

Output from CLINSURV.sas for
Table 2 of Task 53 Statistical Analysis
of Clinical Signs Data Report.

Jennifer R Holdcraft
12/1/2000

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=1 igno=11 IGTYP=3PBIGS

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	110
Noncensored Values	48
Right Censored Values	62
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	-70.18541661

← Col 3
← Col 4

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	5.71892	1.17103	23.8503	<.0001	Intercept
logdose	1	-0.91221	0.22809	15.9943	<.0001	
Scale	1	0.55401	0.06317			Normal scale

← dose response
 slope is highly
 significant - noted
 in report "Results"
 section as
 (p's ≤ 0.0001)

Output from LIFETTD.SAS - Results in
 Table 3.

Jennifer Holdcraft
 12/5/2000

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=1 igno=4 IGTYP=BBIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	30
Noncensored Values	10
Right Censored Values	20
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	-8.754535194

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	15.77683	3.44854	20.9300	<.0001	Intercept
logdose	1	-2.86422	0.66213	18.7121	<.0001	
Scale	1	0.35031	0.08363			Normal scale

Study No. G1555-53A GP CLINICAL SIGNS
MODEL OF time to death - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS
RIGHT-CENSORED REGRESSION OF LOG-GP time to death VS LOG10(GP DOSE)

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=2 igno=12 IGTYPES=PBIGS BBIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	140
Noncensored Values	58
Right Censored Values	82
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	-84.81594687

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	6.78820	1.15486	34.5503	<.0001	Intercept
logdose	1	-1.11810	0.22440	24.8261	<.0001	
Scale	1	0.55233	0.05711			Normal scale

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=2 igno=5 IGTYPES=CIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	40
Noncensored Values	12
Right Censored Values	28
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	-5.50397446

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	2.05628	0.21385	92.4549	<.0001	Intercept
logdose	1	-0.89475	0.18659	22.9960	<.0001	
Scale	1	0.18930	0.04239			Normal scale

Study No. G1555-53A GP CLINICAL SIGNS
 MODEL OF time to death - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS
 DESCRIPTIVE STATISTICS FOR time to death - ANIMALS THAT SHOWED SIGN ONLY

17:42 Thursday, November 30, 2000

PROTYPE	compno	igno	IGTYPE	ntot	nsign	avgdose	avgtttd	stdtttd	ld50	predtime	q25	q75
	1	11	<u>3PBIGS</u>	110	48	<u>167800.26</u>	<u>5.000</u>	<u>2.925</u>	<u>110205.52</u>	<u>13.163</u>	<u>5.568</u>	<u>31.119</u>
		4	<u>BBIG</u>	30	10	<u>178214.12</u>	<u>5.200</u>	<u>3.728</u>	<u>134539.70</u>	<u>12.208</u>	<u>7.086</u>	<u>21.035</u>
A	2	12	<u>PBIGS+BBIG</u>	140	58	<u>169595.75</u>	<u>5.034</u>	<u>3.042</u>	<u>116488.57</u>	<u>13.291</u>	<u>5.637</u>	<u>31.341</u>
		5	<u>CIG</u>	40	12	<u>16.76</u>	<u>8.083</u>	<u>2.883</u>	<u>11.39</u>	<u>12.911</u>	<u>9.622</u>	<u>17.324</u>
						↑	↑	↑	↑	↑	↑	↑
						COL5	COL6	COL7	COL8	COL9		

Table 3 results continued
 Jennifer Holdcraft
 12/5/2000

SEROTYPE=A

no	lpred1	serr1	predtim1	lpred2	serr2	predtim2	diff	serrcomb	zscore	prob
1	1.11935	0.072408	13.1630	1.08666	0.11011	12.2084	0.032694	0.13179	0.24808	<u>0.80407</u>
2	1.12357	0.065545	13.2914	1.11096	0.04967	12.9110	0.012608	0.08224	0.15331	<u>0.87815</u>

↑
 neither is
 significant
 ($p < 0.05$)

P-values determining footnote of

Table 3

Jennifer Holdcraft

12/15/2000

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=1 igno=11 IGTYP=3PBIGS

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	110
Noncensored Values	96
Right Censored Values	14
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	-5.553366097

← Col 3
 ← Col 4

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	3.02014	0.24464	152.4087	<.0001	Intercept
logdose	1	-0.57989	0.04922	138.8000	<.0001	
Scale	1	0.23591	0.01728			Normal scale

← dose response
 slope highly sig.
 Noted in report
 Results section
 p's are
 ≤ 0.0001

Output from LIFETTD.SAS - Results
 in Table 4

Jennifer Holdcraft
 12/5/2000

Study No. G1555-53A GP CLINICAL SIGNS
MODEL OF time to onset of severe signs - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS
RIGHT-CENSORED REGRESSION OF LOG-GP time to onset of severe signs VS LOG10(GP DOSE)

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=1 igno=4 IGTYPESBBIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	30
Noncensored Values	27
Right Censored Values	3
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	7.7499284042

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	3.12979	0.35887	76.0582	<.0001	Intercept
logdose	1	-0.60255	0.07285	68.4106	<.0001	
Scale	1	0.18022	0.02440			Normal scale

SEROTYPE=A compno=2 igno=12 IGTYPES=PBIGS+BBIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	140
Noncensored Values	123
Right Censored Values	17
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	0.7521018629

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	3.03874	0.20597	217.6657	<.0001	Intercept
logdose	1	-0.58376	0.04152	197.6407	<.0001	
Scale	1	0.22473	0.01448			Normal scale

SEROTYPE=A compno=2 igno=5 IGTYPES=CIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	40
Noncensored Values	38
Right Censored Values	2
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	-10.80632237

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	0.85178	0.12660	45.2689	<.0001	Intercept
logdose	1	-0.53325	0.13947	14.6192	0.0001	
Scale	1	0.30185	0.03510			Normal scale

17:42 Thursday, November 30, 2000

DTYPE	compno	igno	IGTYPE	ntot	nsign	avgdose	avgtsev	stdtsev	ld50	predtime	q25	q75
	1	11	<u>3PBIGS</u>	110	96	133808.80	1.495	1.814	110205.52	1.248	0.865	1.800
		4	<u>BBIG</u>	30	27	115254.03	2.148	2.807	134539.70	1.095	0.828	1.449
A	2	12	<u>PBIGS+BBIG</u>	140	123	129735.80	1.638	2.078	116488.57	1.206	0.850	1.709
		5	<u>CIG</u>	40	38	9.63	3.053	2.906	11.39	1.943	1.216	3.104
						↑	↖ ↗	↑	↑	↖ ↗		
						COL5	COL6	COL7	COL8	COL9		

Table 4 results continued
 GRH 12/15/2000

SEROTYPE=A

no	lpred1	serr1	predtim1	lpred2	serr2	predtim2	diff	serrcomb	zscore	prob
1	0.096229	0.023883	1.24804	0.03942	0.038227	1.09503	0.05680	0.045074	1.26023	0.20758
2	0.081239	0.020516	1.20570	0.28839	0.056645	1.94264	-0.20715	0.060246	-3.43844	<u>0.00059</u>

← Significant
 $P < 0.001$
 quoted
 in Results
 section

p-values determining footnote^(a) of Table 4
 GRH 12/5/2000

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=1 igno=11 IGTYPES=3PBIGS

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	110
Noncensored Values	99
Right Censored Values	11
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	34.438980942

← COL 3
 ← COL 4

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	2.55352	0.14711	301.2807	<.0001	Intercept
logdose	1	-0.49914	0.02975	281.5294	<.0001	
Scale	1	0.16184	0.01162			Normal scale

← dose response
 slope is highly
 significant. Noted
 in results section as
 $p < 0.0001$

Output from LIFETTD.sas - Results in Table 5

Gennifer Holdcraft
 12/5/2000

----- SEROTYPE=A doselev1=> LD50 -----

The FREQ Procedure

Table of igtype by incid3

igtype(IGTYPE)

Frequency Row Pct	incid3(Any Sign)	
	Sign Present	Total
<u>PBIGS+BBIG</u>	60 <u>100.00</u>	60
<u>ZCIG</u>	11 <u>100.00</u>	11
Total	71	71

SEROTYPE=A

lpred1	serr1	predtim1	lpred2	serr2	predtim2	diff	serrcomb	zscore	prob
0.036737	0.016368	1.08827	0.02481	0.030366	1.05880	0.01192	0.034496	0.34564	0.72962
0.030490	0.014478	1.07273	0.24840	0.050517	1.77173	-0.21791	0.052551	-4.14660	0.00003

← sig!

$p < 0.0001$
 quoted in
 Results section

P-value determining footnote (a) of

Table 5

GRH 12/5/2000

Study No. G1555-53A GP CLINICAL SIGNS
MODEL OF time to onset of any signs - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS
RIGHT-CENSORED REGRESSION OF LOG-GP time to onset of any signs VS LOG10(GP DOSE)

17:42 Thursday, November 30, 2000

SEROTYPE=A compno=1 igno=4 IGTYPES=BBIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	30
Noncensored Values	28
Right Censored Values	2
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	13.125534284

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	2.32800	0.18288	162.0530	<.0001	Intercept
logdose	1	-0.44907	0.03788	140.5512	<.0001	
Scale	1	0.14609	0.01962			Normal scale

Study No. G1555-53A GP CLINICAL SIGNS
 MODEL OF time to onset of any signs - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS
 RIGHT-CENSORED REGRESSION OF LOG-GP time to onset of any signs VS LOG10(GP DOSE)
 17:42 Thursday, November 30, 2000

SEROTYPE=A compno=2 igno=12 IGTYPES=PBIGS+BBIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	140
Noncensored Values	127
Right Censored Values	13
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	46.778308925

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	2.48167	0.11504	465.3229	<.0001	Intercept
logdose	1	-0.48382	0.02340	427.6276	<.0001	
Scale	1	0.15885	0.01005			Normal scale

Study No. G1555-53A GP CLINICAL SIGNS
 MODEL OF time to onset of any signs - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS
 RIGHT-CENSORED REGRESSION OF LOG-GP time to onset of any signs VS LOG10(GP DOSE)
 17:42 Thursday, November 30, 2000

SEROTYPE=A compno=2 igno=5 IGTYP=E=CIG

The LIFEREG Procedure

Model Information

Data Set	WORK.D4
Dependent Variable	ltimons1
Dependent Variable	ltimons2
Number of Observations	40
Noncensored Values	38
Right Censored Values	2
Left Censored Values	0
Interval Censored Values	0
Missing Values	122
Name of Distribution	NORMAL
Log Likelihood	-6.70146459

Algorithm converged.

Analysis of Parameter Estimates

Variable	DF	Estimate	Standard Error	Chi-Square	Pr > ChiSq	Label
Intercept	1	0.79138	0.11252	49.4699	<.0001	Intercept
logdose	1	-0.51394	0.12405	17.1650	<.0001	
Scale	1	0.26920	0.03137			Normal scale

Study No. G1555-53A GP CLINICAL SIGNS
 MODEL OF time to onset of any signs - USE LD50s FOR POOLED IGTYPES TO COMPARE GROUPS
 DESCRIPTIVE STATISTICS FOR time to onset of any signs - ANIMALS THAT SHOWED SIGN ONLY
 17:42 Thursday, November 30, 2000

PE	compno	igno	IGTYPE	ntot	nsign	avgdose	avgtany	stdtany	ld50	predtime	q25	q75
	1	11	<u>3PBIGS</u>	110	99	130825.84	1.298	1.340	110205.52	1.088	0.846	1.399
		4	<u>BBIG</u>	30	28	111144.32	2.107	2.869	134539.70	1.059	0.844	1.328
	2	12	<u>PBIGS+BBIG</u>	140	127	126486.60	1.476	1.809	116488.57	1.073	0.838	1.373
		5	<u>CIG</u>	40	38	9.63	2.605	2.521	11.39	1.772	1.166	2.691
						↑	↑	↑	↑	↑	↑	↑
						COL5	COL6	COL7	COL8	COL9		

Table 5 results continued

QRH 12/5/2000

The CORR Procedure

2 Variables: durany dursev

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum	Label
durany	180	23.07222	8.49517	4153	0	28.00000	Duration of any sign
dursev	180	18.28889	10.35757	3292	0	28.00000	Duration of severe signs

Pearson Correlation Coefficients, N = 180
Prob > |r| under H0: Rho=0

	durany	dursev
durany Duration of any sign	1.00000	0.67957 <.0001
dursev Duration of severe signs	0.67957 <.0001	1.00000

→ 0.68
p < 0.0001

Output from CORRTIME.sas
- Result quoted in report "Methods" section

Jennifer Holdcraft
12/5/2000

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYPE

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
19:40 Tuesday, December 5, 2000

SEROTYPE=A compno=1 igno=11 IGTYPE=3PBIGS

The GENMOD Procedure

Model Information

Data Set	WORK.DALL29
Distribution	Binomial
Link Function	Probit
Response Variable (Events)	indsign
Response Variable (Trials)	nint
Observations Used	3080
Number Of Events	2587
Number Of Trials	3080
Missing Values	1

Class Level Information

Class	Levels	Values
GPID	111	14 18 11 46 4 47 12 40 39 49 3 23 38 42 45 91 63 129 99 96 93 108 90 104 24 19 41 282 136 261 250 119 110 65 80 117 115 133 89 268 262 284 264 266 241 251 151 239 265 219 218 189 213 191 280 248 255 242 259 126 101 254 166 246 69 210 243 97 83 197 145 ...

Parameter Information

Parameter	Effect
Prm1	Intercept
Prm2	logdose

Criteria For Assessing Goodness Of Fit

Criterion	DF	Value	Value/DF
Deviance	3078	1211.5605	0.3936
Scaled Deviance	3078	1211.5605	0.3936
Pearson Chi-Square	3078	4753.4053	1.5443
Scaled Pearson X2	3078	4753.4053	1.5443
Log Likelihood		-605.7803	

Algorithm converged.

Analysis Of Initial Parameter Estimates

Parameter	DF	Estimate	Standard Error	Wald 95% Confidence Limits	Chi-Square	Pr > ChiSq
Intercept	1	-6.3804	0.3231	-7.0136 -5.7472	390.04	<.0001
logdose	1	1.6159	0.0685	1.4816 1.7501	556.42	<.0001
Scale	0	1.0000	0.0000	1.0000 1.0000		

NOTE: The scale parameter was held fixed.

GEE Model Information

Correlation Structure	Exchangeable
Subject Effect	GPID (111 levels)
Number of Clusters	111
Clusters With Missing Values	1

GENMOD output
from GENMODRN.sas
→ p-values quoted
in results section
Jennifer Haddcraft
12/5/2000

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTTYPE

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
19:40 Tuesday, December 5, 2000

----- SEROTYPE=A compno=1 igno=11 IGTTYPE=3PBIGS -----

The GENMOD Procedure

Algorithm converged.

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter Estimate	Standard Error	95% Confidence Limits		Z	Pr > Z
Intercept	-6.3804	0.6862	-7.7254 -5.0354	-9.30	<.0001
logdose	1.6159	0.1388	1.3438 1.8879	11.64	<.0001

*dose response
slope is highly
significant
 $p < 0.0001$*

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYPE

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT

19:40 Tuesday, December 5, 2000

SEROTYPE=A compno=1 igno=4 IGTYPE=BBIG

The GENMOD Procedure

Model Information

Data Set	WORK.DALL29
Distribution	Binomial
Link Function	Probit
Response Variable (Events)	indsign
Response Variable (Trials)	nint
Observations Used	840
Number Of Events	682
Number Of Trials	840
Missing Values	1

Class Level Information

Class	Levels	Values
GPID	31	36 6 34 35 15 114 87 95 2 130 131 134 164 167 156 127 135 116 212 209 214 196 170 207 223 149 179 75 60 141 1001

Parameter Information

Parameter	Effect
Prm1	Intercept
Prm2	logdose

Criteria For Assessing Goodness Of Fit

Criterion	DF	Value	Value/DF
Deviance	838	348.1937	0.4155
Scaled Deviance	838	348.1937	0.4155
Pearson Chi-Square	838	1368.0625	1.6325
Scaled Pearson X2	838	1368.0625	1.6325
Log Likelihood		-174.0968	

Algorithm converged.

Analysis Of Initial Parameter Estimates

Parameter	DF	Estimate	Standard Error	Wald 95% Confidence Limits	Chi-Square	Pr > ChiSq
Intercept	1	-6.4063	0.5644	-7.5124 -5.3001	128.85	<.0001
logdose	1	1.6192	0.1208	1.3824 1.8560	179.63	<.0001
Scale	0	1.0000	0.0000	1.0000 1.0000		

NOTE: The scale parameter was held fixed.

GEE Model Information

Correlation Structure	Exchangeable
Subject Effect	GPID (31 levels)
Number of Clusters	31
Clusters With Missing Values	1
Correlation Matrix Dimension	28
Maximum Cluster Size	28
Minimum Cluster Size	0

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYP

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
19:40 Tuesday, December 5, 2000

----- SEROTYPE=A compno=1 igno=4 IGTYP=BBIG -----

The GENMOD Procedure

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter Estimate	Standard Error	95% Confidence Limits		Z	Pr > Z
Intercept	-6.4063	0.9697	-8.3069 -4.5056	-6.61	<.0001
logdose	1.6192	0.2001	1.2270 2.0114	8.09	<.0001

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYP

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
19:40 Tuesday, December 5, 2000

SEROTYPE=A compno=2 igno=12 IGTYP=PBIGS+BBIG

The GENMOD Procedure

Model Information

Data Set	WORK.DALL29
Distribution	Binomial
Link Function	Probit
Response Variable (Events)	indsign
Response Variable (Trials)	nint
Observations Used	3920
Number Of Events	3269
Number Of Trials	3920
Missing Values	1

Class Level Information

Class	Levels	Values
GPID	141	14 18 11 36 46 6 4 47 12 40 39 34 49 3 35 23 38 42 15 45 91 63 129 114 99 87 96 95 93 108 90 104 2 24 19 41 282 136 261 250 119 110 130 131 134 65 80 117 115 133 89 268 262 284 264 266 241 251 151 239 265 164 167 219 156 218 189 213 191 280 248 255 242 ...

Parameter Information

Parameter	Effect
Prm1	Intercept
Prm2	logdose

Criteria For Assessing Goodness Of Fit

Criterion	DF	Value	Value/DF
Deviance	3918	1559.7710	0.3981
Scaled Deviance	3918	1559.7710	0.3981
Pearson Chi-Square	3918	6105.8569	1.5584
Scaled Pearson X2	3918	6105.8569	1.5584
Log Likelihood		-779.8855	

Algorithm converged.

Analysis Of Initial Parameter Estimates

Parameter	DF	Estimate	Standard Error	Wald 95% Confidence Limits	Chi-Square	Pr > ChiSq
Intercept	1	-6.3878	0.2803	-6.9372 -5.8385	519.39	<.0001
logdose	1	1.6170	0.0596	1.5002 1.7337	737.12	<.0001
Scale	0	1.0000	0.0000	1.0000 1.0000		

NOTE: The scale parameter was held fixed.

GEE Model Information

Correlation Structure	Exchangeable
Subject Effect	GPID (141 levels)
Number of Clusters	141
Clusters With Missing Values	1

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYPE

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
19:40 Tuesday, December 5, 2000

----- SEROTYPE=A compno=2 igno=12 IGTYPE=PBIGS+BBIG -----

The GENMOD Procedure

Algorithm converged.

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter	Estimate	Standard Error	95% Confidence Limits		Z	Pr > Z
Intercept	-6.3878	0.5700	-7.5051	-5.2706	-11.21	<.0001
logdose	1.6170	0.1157	1.3902	1.8437	13.98	<.0001

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYPE

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
19:40 Tuesday, December 5, 2000

SEROTYPE=A compno=2 igno=5 IGTYPE=CIG

The GENMOD Procedure

Model Information

Data Set	WORK.DALL29
Distribution	Binomial
Link Function	Probit
Response Variable (Events)	indsign
Response Variable (Trials)	nint
Observations Used	1120
Number Of Events	884
Number Of Trials	1120
Missing Values	1

Class Level Information

Class	Levels	Values
GPID	41	8 71 100 98 9 76 84 70 137 59 68 48 237 273 274 276 269 253 118 271 260 142 277 94 283 190 204 154 50 235 174 177 183 232 216 173 53 206 187 31 1003

Parameter Information

Parameter	Effect
Prm1	Intercept
Prm2	logdose

Criteria For Assessing Goodness Of Fit

Criterion	DF	Value	Value/DF
Deviance	1118	934.2084	0.8356
Scaled Deviance	1118	934.2084	0.8356
Pearson Chi-Square	1118	1685.8707	1.5079
Scaled Pearson X2	1118	1685.8707	1.5079
Log Likelihood		-467.1042	

Algorithm converged.

Analysis Of Initial Parameter Estimates

Parameter	DF	Estimate	Standard Error	Wald 95% Confidence Limits		Chi-Square	Pr > ChiSq
Intercept	1	-0.6591	0.1130	-0.8806	-0.4376	34.01	<.0001
logdose	1	1.9290	0.1417	1.6514	2.2067	185.40	<.0001
Scale	0	1.0000	0.0000	1.0000	1.0000		

NOTE: The scale parameter was held fixed.

GEE Model Information

Correlation Structure	Exchangeable
Subject Effect	GPID (41 levels)
Number of Clusters	41
Clusters With Missing Values	1
Correlation Matrix Dimension	28
Maximum Cluster Size	28
Minimum Cluster Size	0

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYP

PROC GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
19:40 Tuesday, December 5, 2000

----- SEROTYPE=A compno=2 igno=5 IGTYP=CIG -----

The GENMOD Procedure

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter	Estimate	Standard Error	95% Confidence Limits		Z	Pr > Z
Intercept	-0.6591	0.3755	-1.3951	0.0769	-1.76	0.0792
logdose	1.9290	0.4338	1.0789	2.7792	4.45	<.0001

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYPE

GENMOD ON proportion of time showing any sign VS LOG10(GP DOSE) TO GET PROBIT MODEL WITH RANDOM SUBJECT EFFECT
PREDICTED VALUES AT THE LD50 DOSES

14:26 Friday, December 1, 2000

	G	P	l	c	p	X	s	L	U	p	l	u	
	D	i	o	o	r	b	t	o	p	e	o	p	
	O	r	d	i	d	e	d	w	p	d	u	d	
	S	e	o	g	p	t	e	e	p	u	u	u	
	E	d	e	n	r	a	a	r	r	n	n	r	
PBIGS	110206	1	5.042	11	1	0.9614	1.7672	0.0493398	0.9525889	0.9688321	13.4596	13.3362	13.5636
BBIG	134540	1	5.129	4	1	0.9712	1.8983	0.0917894	0.957136	0.9811538	13.5964	13.3999	13.7362
PBIGS+BBIG	116489	1	5.066	12	2	0.9644	1.8042	0.0436071	0.9571676	0.9705983	13.5016	13.4003	13.5884
IG	11.39	1	1.057	5	2	0.9160	1.3790	0.1363086	0.8668927	0.9501328	12.8247	12.1365	13.3019

col 7

↑
COL 8

↑ ↑
COL 9

Output listing from GENMODRN.SAS
Results in Table 6.

Jennifer R Holdcraft
12/5/2000

TASK 53 DURATION OF CLINICAL SIGNS - MODEL FOR EACH SEROTYPE AND IGTYP
 DESCRIPTIVE STATS FOR ANIMALS THAT SHOWED SIGNS

14:26 Friday, December 1, 2000

SEROTYPE	compno	IGTYPE	ntot	nsign	avgdose	stddose	avgdurn	stdurn
A	1	3PBIGS	110	99	130825.84	88845.96	13.0657	1.75785
		BBIG	30	28	111144.32	74136.83	12.1786	3.55438
A	2	PBIGS+BBIG	140	127	126486.60	85932.21	12.8701	2.29061
		CIG	40	38	9.63	7.62	11.6316	3.05730

↑ ↑ ↑
 COL3 COL4 COL5

↑ ↑
 COL6

SEROTYPE=A

compno	ld501	xbeta1	serr1	pred1	ld502	xbeta2
1	110206	1.7672	0.049340	0.9614 (0.9526, 0.9688)	134540	1.8983
2	116489	1.8042	0.043607	0.9644 (0.9572, 0.9706)	11.39	1.3790
serr2	pred2	diff	serrcomb	zscore	prob	
0.09179	0.9712 (0.9571, 0.9812)	-0.13108	0.10421	-1.258	0.20843	
0.13631	0.9160 (0.8669, 0.9501)	0.42521	0.14311	2.971	0.00297	

significant
 $p < 0.005$

p-value determining footnote (a)
of Table 6 GRH 12/5/2000

----- IGTYPE=BBIG total=30 -----

Obs	SEROTYPE	SIGNNO	CLINSIGN	nsign	percent	outpct
1	A	2	Ruffled fur	17	56.7	17 (57)
2	A	3	Lab breathing	13	43.3	13 (43)
3	A	4	Droopy eyes	5	16.7	5 (17)
4	A	5	Weak limbs	26	86.7	26 (87)
5	A	6	Salivation	10	33.3	10 (33)
6	A	7	Lacrimation	5	16.7	5 (17)
7	A	8	Hindlimb paralysis	23	76.7	23 (77)
8	A	9	Total paralysis	3	10.0	3 (10)
9	A	10	Other signs	15	50.0	15 (50)
10	A	11	Dead	10	33.3	10 (33)

Output from
FREQCOMM.sas.
Results in 1st 10
rows Table 7
Jennifer Heldercraft
12/15/2000

----- IGTYPE=CIG total=40 -----

Obs	SEROTYPE	SIGNNO	CLINSIGN	nsign	percent	outpct
11	A	2	Ruffled fur	31	77.5	31 (78)
12	A	3	Lab breathing	18	45.0	18 (45)
13	A	4	Droopy eyes	6	15.0	6 (15)
14	A	5	Weak limbs	38	95.0	38 (95)
15	A	6	Salivation	15	37.5	15 (38)
16	A	7	Lacrimation	12	30.0	12 (30)
17	A	8	Hindlimb paralysis	35	87.5	35 (88)
18	A	9	Total paralysis	4	10.0	4 (10)
19	A	10	Other signs	19	47.5	19 (48)
20	A	11	Dead	12	30.0	12 (30)

↑ values
in table

----- IGTYPE=PBIGA total=40 -----

Obs	SEROTYPE	SIGNNO	CLINSIGN	nsign	percent	outpct
21	A	2	Ruffled fur	30	75.0	30 (75)
22	A	3	Lab breathing	21	52.5	21 (53)
23	A	4	Droopy eyes	11	27.5	11 (28)
24	A	5	Weak limbs	34	85.0	34 (85)
25	A	6	Salivation	21	52.5	21 (53)
26	A	7	Lacrimation	18	45.0	18 (45)
27	A	8	Hindlimb paralysis	34	85.0	34 (85)
28	A	9	Total paralysis	4	10.0	4 (10)
29	A	10	Other signs	16	40.0	16 (40)
30	A	11	Dead	19	47.5	19 (48)

----- IGTYPE=PBIGAB total=40 -----

Obs	SEROTYPE	SIGNNO	CLINSIGN	nsign	percent	outpct
31	A	2	Ruffled fur	30	75.0	30 (75)
32	A	3	Lab breathing	25	62.5	25 (63)
33	A	4	Droopy eyes	11	27.5	11 (28)
34	A	5	Weak limbs	35	87.5	35 (88)
35	A	6	Salivation	19	47.5	19 (48)
36	A	7	Lacrimation	15	37.5	15 (38)
37	A	8	Hindlimb paralysis	32	80.0	32 (80)
38	A	9	Total paralysis	3	7.5	3 (8)
39	A	10	Other signs	18	45.0	18 (45)
40	A	11	Dead	20	50.0	20 (50)

----- IGTYPE=PBIGB total=30 -----

Obs	SEROTYPE	SIGNNO	CLINSIGN	nsign	percent	outpct
41	A	2	Ruffled fur	15	50.0	15 (50)
42	A	3	Lab breathing	10	33.3	10 (33)
43	A	4	Droopy eyes	5	16.7	5 (17)
44	A	5	Weak limbs	24	80.0	24 (80)
45	A	6	Salivation	9	30.0	9 (30)
46	A	7	Lacrimation	5	16.7	5 (17)
47	A	8	Hindlimb paralysis	25	83.3	25 (83)

→ cont.

----- IGTYPE=PBIGB total=30 -----

(continued)

Obs	SEROTYPE	SIGNNO	CLINSIGN	nsign	percent	outpct
48	A	9	Total paralysis	2	6.7	2 (7)
49	A	10	Other signs	10	33.3	10 (33)
50	A	11	Dead	9	30.0	9 (30)

14:33 Friday, December 1, 2000

TASK 53 CLINICAL SIGNS - OTHER SYMPTOMS (GROUPED)
 FREQUENCIES AND PERCENTS OF ANIMALS SHOWING SIGNS AT LEAST ONCE

SEROTYPE=A IGTYPE=BBIG total=30

Obs	signno	clinsign	nsign	percent	outpct
1	13	DEHYDRATED	8	26.7	8 (27)
2	14	DIARRHEA	12	40.0	12 (40)
3	16	MORIBUND/EUTHANIZED	4	13.3	4 (13)

SEROTYPE=A IGTYPE=CIG total=40

Obs	signno	clinsign	nsign	percent	outpct
4	12	BLOODY DIARRHEA/BLEEDING FROM HIND END	2	5.0	2 (5)
5	13	DEHYDRATED	9	22.5	9 (23)
6	14	DIARRHEA	17	42.5	17 (43)
7	15	HAIR LOSS	1	2.5	1 (3)
8	16	MORIBUND/EUTHANIZED	4	10.0	4 (10)
9	17	PROLAPSED RECTUM/PROLAPSED PENIS	2	5.0	2 (5)

SEROTYPE=A IGTYPE=PBIGA total=40

Obs	signno	clinsign	nsign	percent	outpct
10	13	DEHYDRATED	5	12.5	5 (13)
11	14	DIARRHEA	16	40.0	16 (40)
12	16	MORIBUND/EUTHANIZED	11	27.5	11 (28)
13	17	PROLAPSED RECTUM/PROLAPSED PENIS	1	2.5	1 (3)

SEROTYPE=A IGTYPE=PBIGAB total=40

Obs	signno	clinsign	nsign	percent	outpct
14	12	BLOODY DIARRHEA/BLEEDING FROM HIND END	1	2.5	1 (3)
15	13	DEHYDRATED	9	22.5	9 (23)
16	14	DIARRHEA	14	35.0	14 (35)
17	15	HAIR LOSS	1	2.5	1 (3)
18	16	MORIBUND/EUTHANIZED	13	32.5	13 (33)

SEROTYPE=A IGTYPE=PBIGB total=30

Obs	signno	clinsign	nsign	percent	outpct
19	12	BLOODY DIARRHEA/BLEEDING FROM HIND END	1	3.3	1 (3)
20	13	DEHYDRATED	4	13.3	4 (13)
21	14	DIARRHEA	8	26.7	8 (27)
22	16	MORIBUND/EUTHANIZED	6	20.0	6 (20)
23	17	PROLAPSED RECTUM/PROLAPSED PENIS	1	3.3	1 (3)
24	18	RUNNY NOSE	1	3.3	1 (3)

↑ values in table

Output from FREQCOMM.sas
 Results in 2nd half of Table 7

Jennifer Holdcraft

APPENDIX F

Chemistry Reports for Monomeric IgG of the Purified, Lyophilized Immunoglobulins

Date November 15, 1999

To File

From Jon Kohne *JWK 11-15-99*

Subject G1555-53A FPLC Analyses performed on
November 12, 1999

The data package for Task 53 FPLC analyses performed on November 12, 1999 consists of an eleven page report addressed to Robert Hunt, and FPLC data from November 12, 1999-47 pages.

QC'd by:

Ted Miller 11-16-99

Reviewed by:

Tim Hays 12-8-99

INTERNAL CHEMISTRY REPORT

from

Chemistry

on

Task 53

Project Number G155553A

to

Robert Hunt

November 15, 1999

by

Jon Kohne

Timothy Hayes

BATTELLE

Medical Research and Evaluation Facility

505 King Avenue, Building JM-3

Columbus, Ohio 43201-2693

Introduction

The chemistry group at the Medical Research and Evaluation Facility (MREF) was asked to evaluate the relative composition of components in antibody preparations. The analytical method was based on that published in: Botulism Immune Globulin (Human), Pentavalent, Lot 1A, 1B and 2A: Storage and Stability Report, The Salk Institute, July, 1996.

Experimental

The samples of lyophilized gammaglobulin or CIG were analyzed by modified FPLC (Fast Protein Liquid Chromatography) using a Waters Alliance Liquid Chromatograph equipped with a Waters 996 photodiode array detector (scan 200-600 nm, quantitate 280 nm). The separation was accomplished using a Superdex 200 HR column (Supelco, Bellefonte, PA, part number 17-1088-01) with 50 mM sodium phosphate buffer (pH 7) mobile phase. Known standards of antibody fragments IgG, F(ab')₂, Fc, and Fab (Calbiochem, La Jolla, CA) were chromatographed for comparison of retention times. The F(ab')₂ was received in a solution with the concentration listed as 2.49 mg/mL protein. The IgG, Fab and Fc were received as dry powders. The IgG and Fab were diluted to a nominal concentration of 2 mg/mL with mobile phase using the vendors assigned weight documented on the vial. The Fc was diluted to a concentration of 2.5 mg/mL with mobile phase also using the vendors assigned weight documented on the vial. A detailed description of the analytical procedure is contained in Chemistry Method No. 14, February 13, 1998.

To investigate the effects of freezing, a sample of the IgG standard was analyzed with storage at room temperature and after being stored at -20°C.

Standards

Standards were prepared from the following lyophilized material purchased from Calbiochem:

1. Human Immunoglobulin G, Plasma, IgG, Calbiochem, catalog number 401114, lot number B23973, sample number 394-20-06.
2. Human Immunoglobulin G, Fab Fragment, Plasma, Calbiochem, catalog number 401116, lot number B14394, sample number 394-19-21.
3. Human Immunoglobulin G, F(ab')₂ Fragment, Plasma, Calbiochem, catalog number 401103, lot number B22818, sample number 394-21-04.
4. Human Immunoglobulin G, Fc Fragment, Plasma, Calbiochem, catalog number 401104, lot number B27070, sample number 394-20-21.

Samples

The following samples were received for analysis. Approximately 2 mg of powder was weighed into a 1.0-mL volumetric flask and diluted to volume with mobile phase.

1. Lyophilized gammaglobulin 09/24/99; Bottle 1; sample number 394-22-05.
2. Lyophilized gammaglobulin 09/24/99; Bottle 2; sample number 394-22-09.
3. Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 1; sample number 394-22-13.
4. Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 2; sample number 394-22-18.
5. Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 5; sample number 394-22-23.

Results

Tables 1-9 contain a list of the retention times and the relative percent area of peaks in the chromatograms of the IgG, Fab, F(ab')₂, Fc and samples. Chromatograms 1-9 are included after each corresponding table. The samples and standards were run in duplicate to verify reproducibility, however, the chromatograms and tables presented contain data from the first injection only. The %RSD was less than 1.0% for the peak areas of the sample and standards.

Table 10 and chromatogram 10 are the results of the analysis of the Human Immunoglobulin G standard in mobile phase after storage at -20 C.

Discussion

The lyophilized gammaglobulin samples contained 96% or better of the recorded chromatographic response at the same retention time as the IgG monomer standard. The average area percent of this peak for the samples received September 24, 1999 was 97.3 percent. The average area percent of this peak for the samples received October 14, 1999, Lot D1060 was 97.9 percent.

In analyzing these samples, an observation was made that the storage of the IgG standards at -20 C resulted in a degradation of the monomer peak. This is reflected in the comparison of chromatogram 1 (room temperature storage) to chromatogram 10 (-20 C storage). The data shows that the freeze/thaw cycle causes degradation of the IgG monomer.

Conclusions

The lyophilized gammaglobulin analyzed has a chromatographic purity greater than 95%. The samples and calibration standards should be prepared fresh and kept at room temperature for each analysis, unless a storage condition can be confirmed that does not result in monomer degradation. The storage condition of -20 C has been shown to yield a significant degradation of the monomer component.

Table 1. IgG 394-20-06 2 mg/mL Lot B23973 room temp storage

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	1.59	1.57	21.66
IgG,Monomer	98.41	97.35	24.64
Low molecular weight components	-	1.07	31.06

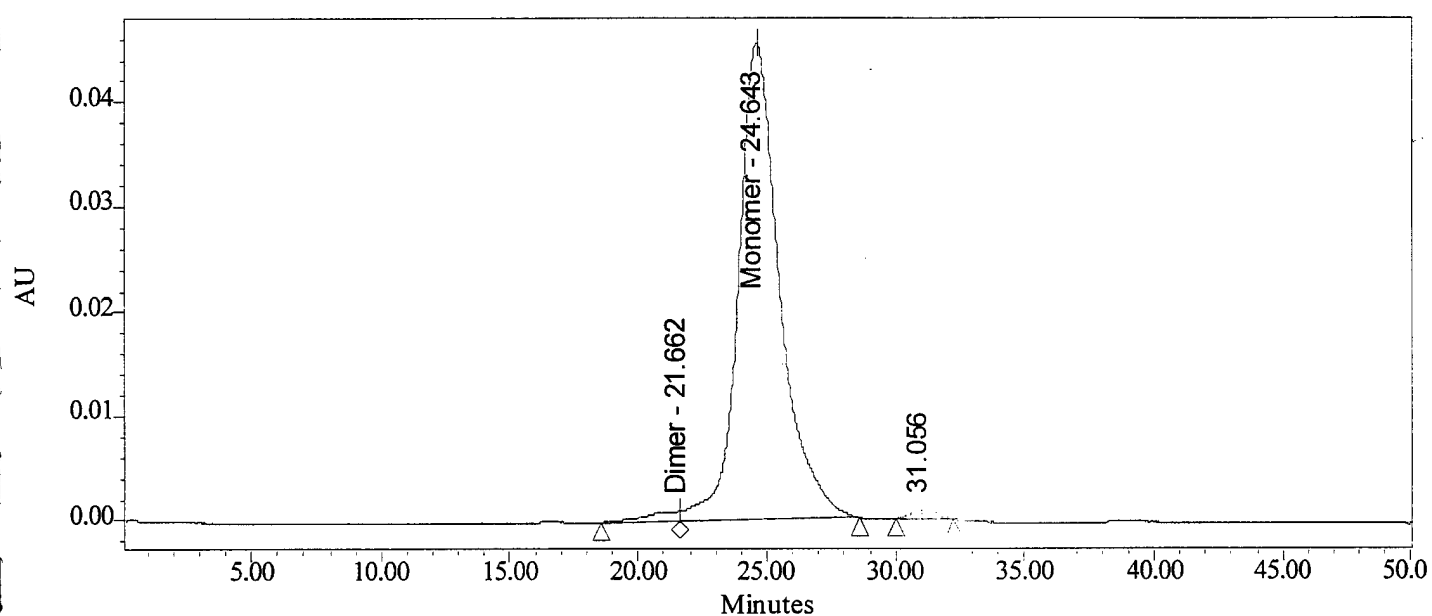
Figure 1. IgG 394-20-06 2 mg/mL Lot B23973 room temp storage

Table 2. Sample 1 - Lyophilized gammaglobulin Rec'd 09/24/99 394-22-05

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.029	0.29	16.71
IgG,Dimer	2.55	2.54	21.55
IgG,Monomer	97.16	96.51	24.57
Low molecular weight components	-	0.19	38.59
Low molecular weight components	-	0.47	40.61

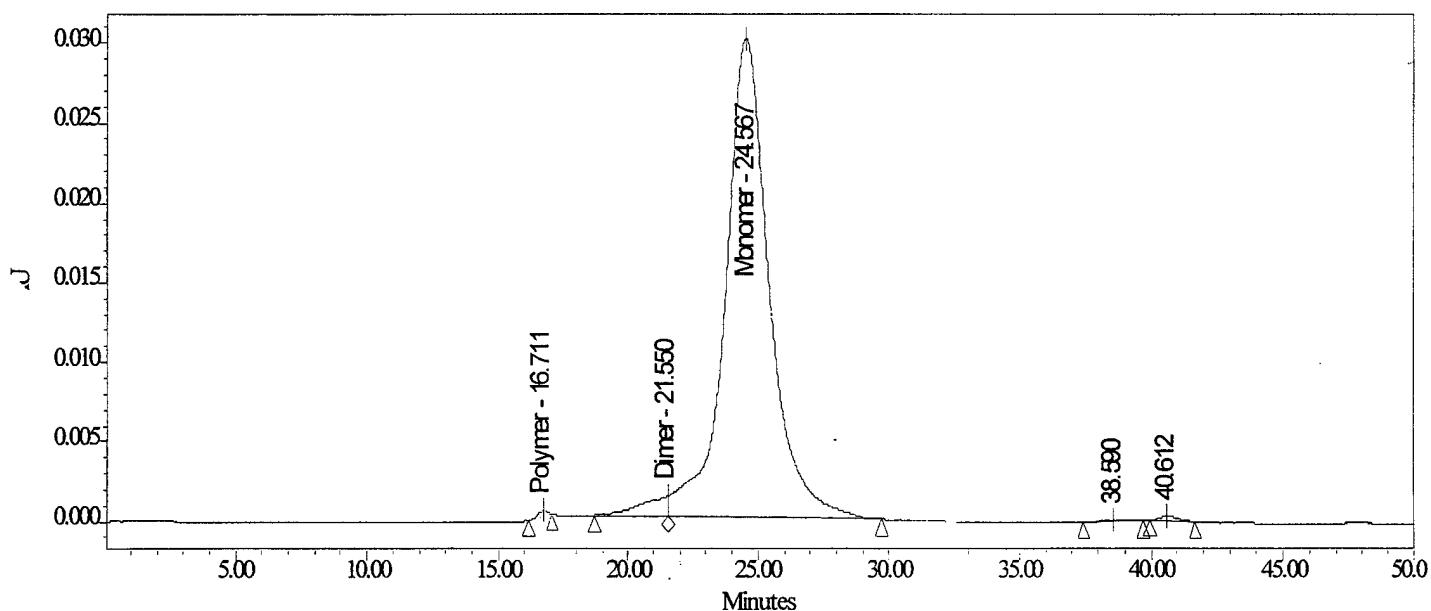
Figure 2. Sample 1 - Lyophilized gammaglobulin Rec'd 09/24/99 394-22-05

Table 3. Sample 2- Lyophilized gammaglobulin Rec'd 09/24/99 394-22-09

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.27	0.27	16.70
IgG,Dimer	2.21	2.19	21.59
IgG,Monomer	97.52	96.86	24.56
Low molecular weight components	-	0.20	38.58
Low molecular weight components	-	0.48	40.63

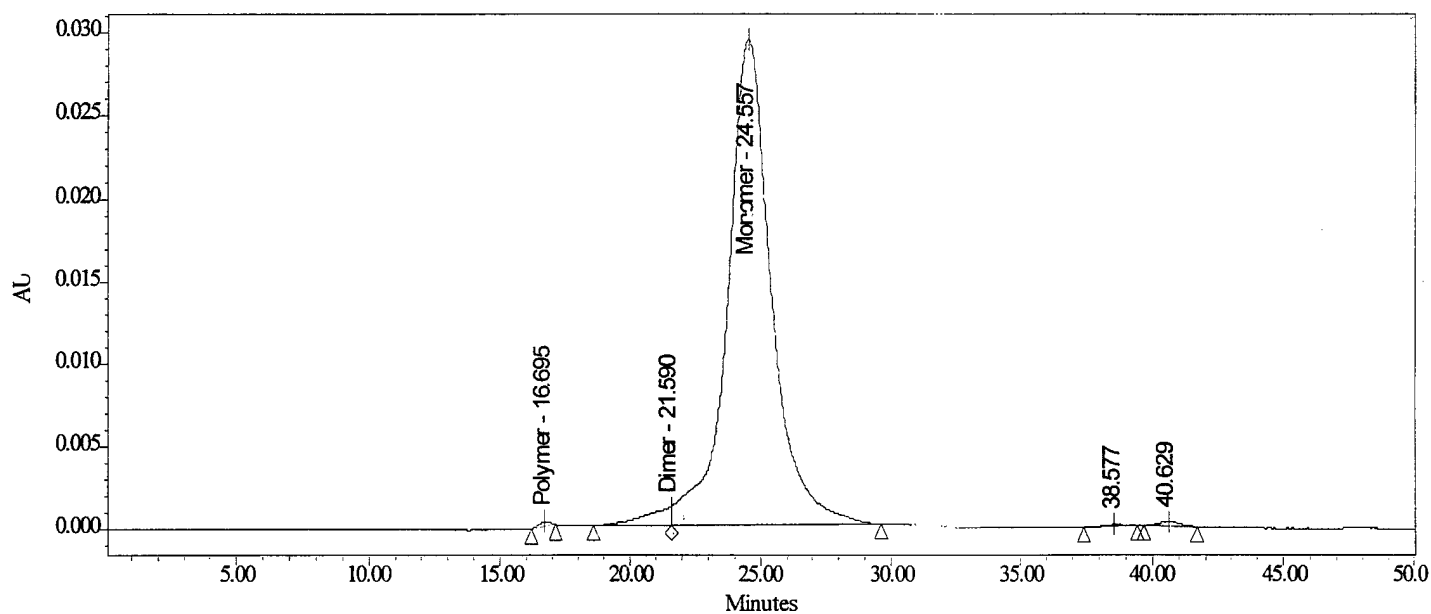
Figure 3. Sample 2- Lyophilized gammaglobulin Rec'd 09/24/99 394-22-09

Table 4. Sample 3- Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 1

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.43	0.43	16.63
IgG,Dimer	1.28	1.27	21.57
IgG,Monomer	97.84	96.73	24.57
Low molecular weight components	-	0.39	38.47
Low molecular weight components	-	1.19	40.65

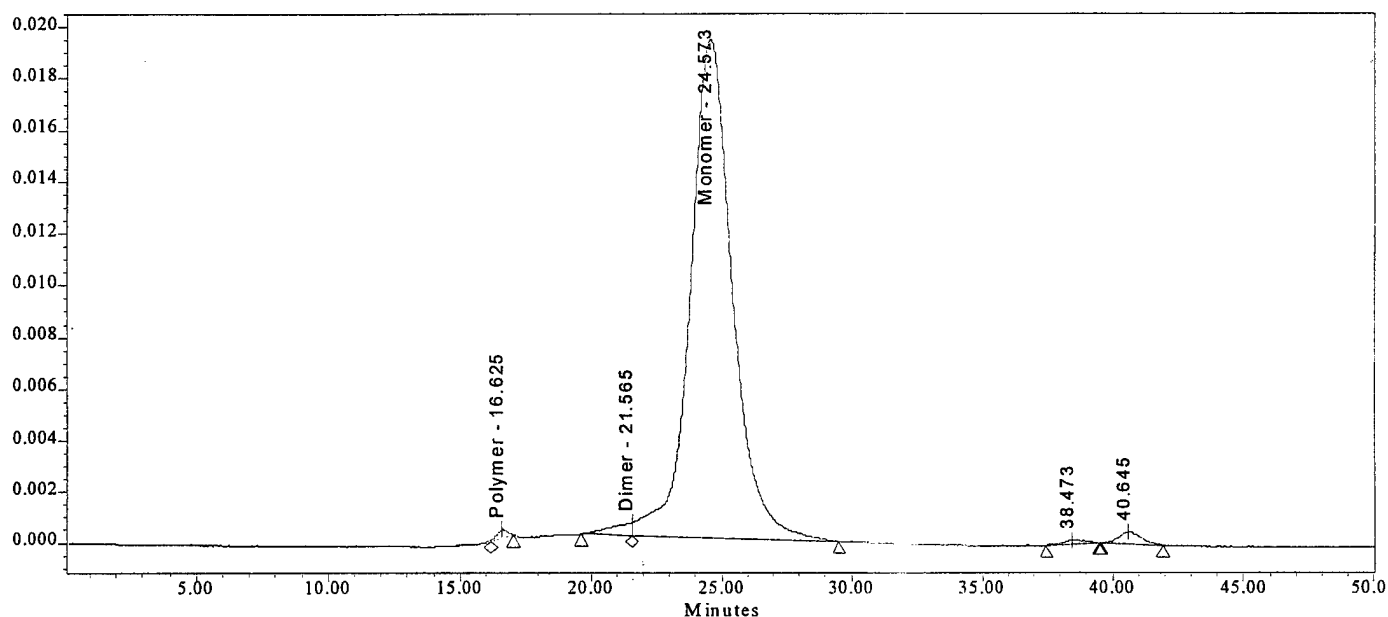
Figure 4. Sample 3- Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 1

Table 5. Sample 4- Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 2

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.65	0.64	16.61
IgG,Dimer	1.32	1.31	21.57
IgG,Monomer	98.02	96.58	24.57
Low molecular weight components	-	0.35	38.48
Low molecular weight components	-	1.12	40.65

Figure 5. Sample 4- Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 2

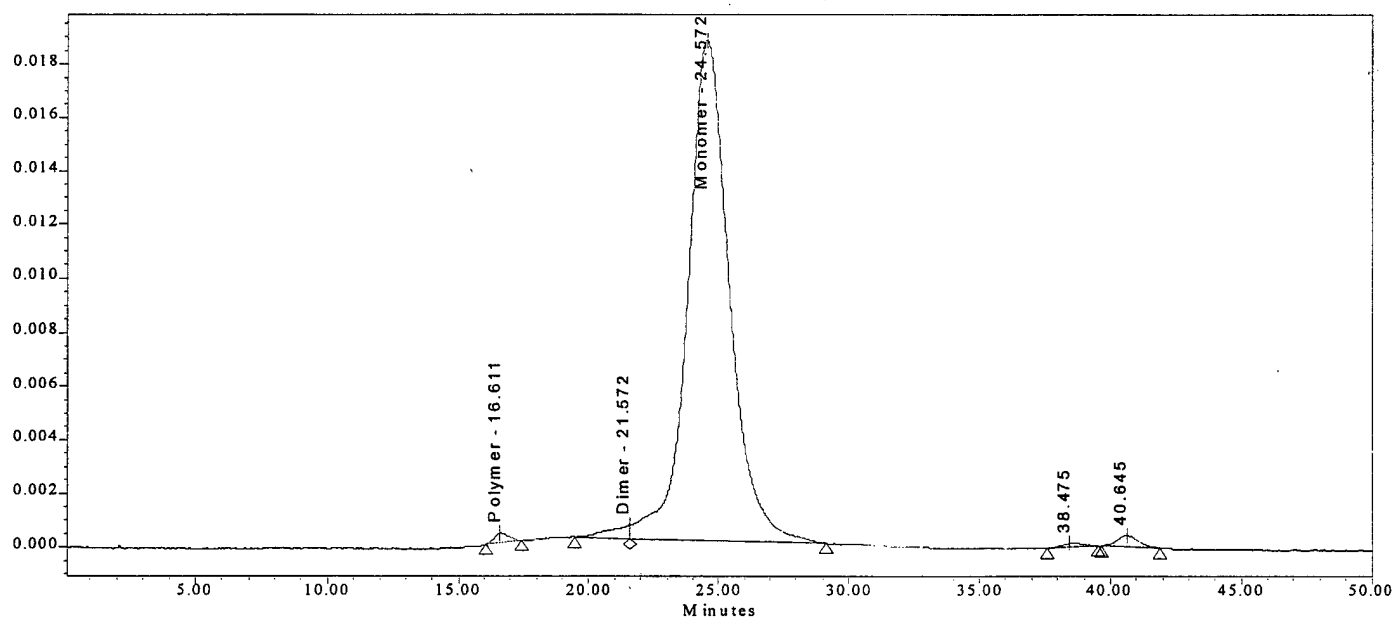


Table 6. Sample 5- Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 5

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.69	0.68	16.57
IgG,Dimer	1.47	1.45	21.58
IgG,Monomer	97.83	96.38	24.57
Low molecular weight components	-	0.36	38.61
Low molecular weight components	-	1.13	40.70

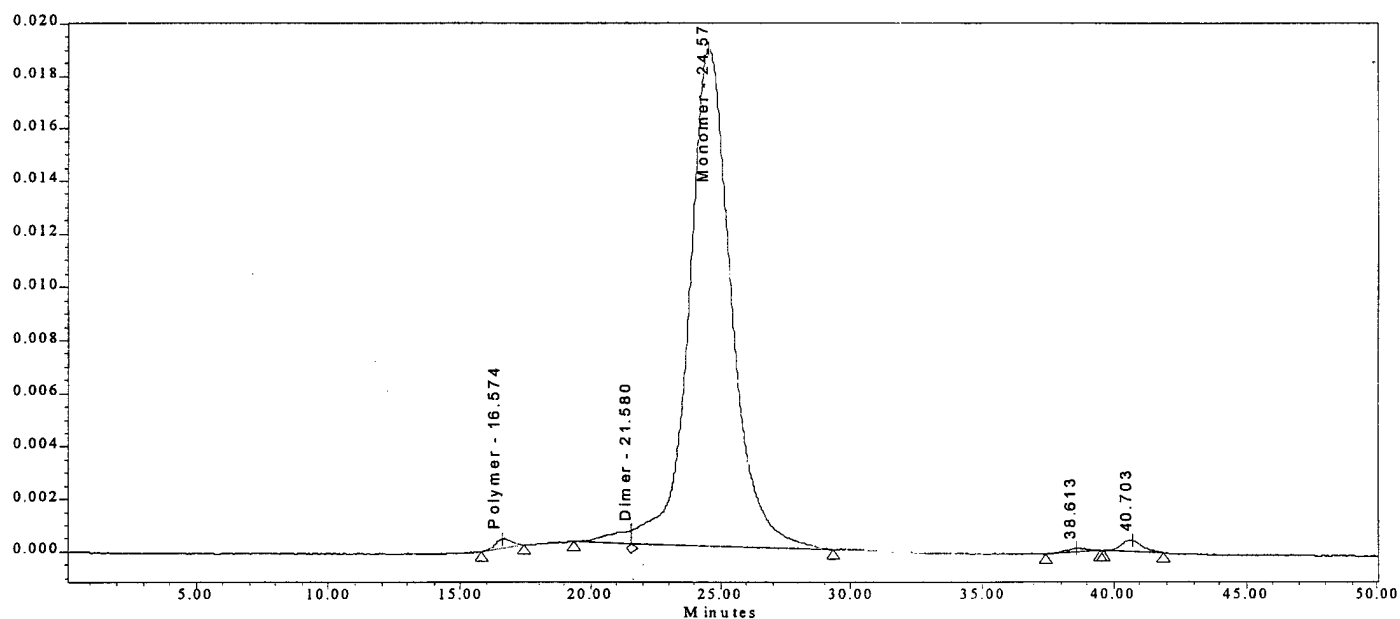
Figure 6. Sample 5- Lyophilized gammaglobulin 10/14/99, Lot D1060; Box 5

Table 7. Fab Lot B14394, 394-19-21

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
F(ab)'2	-	100.00	30.95

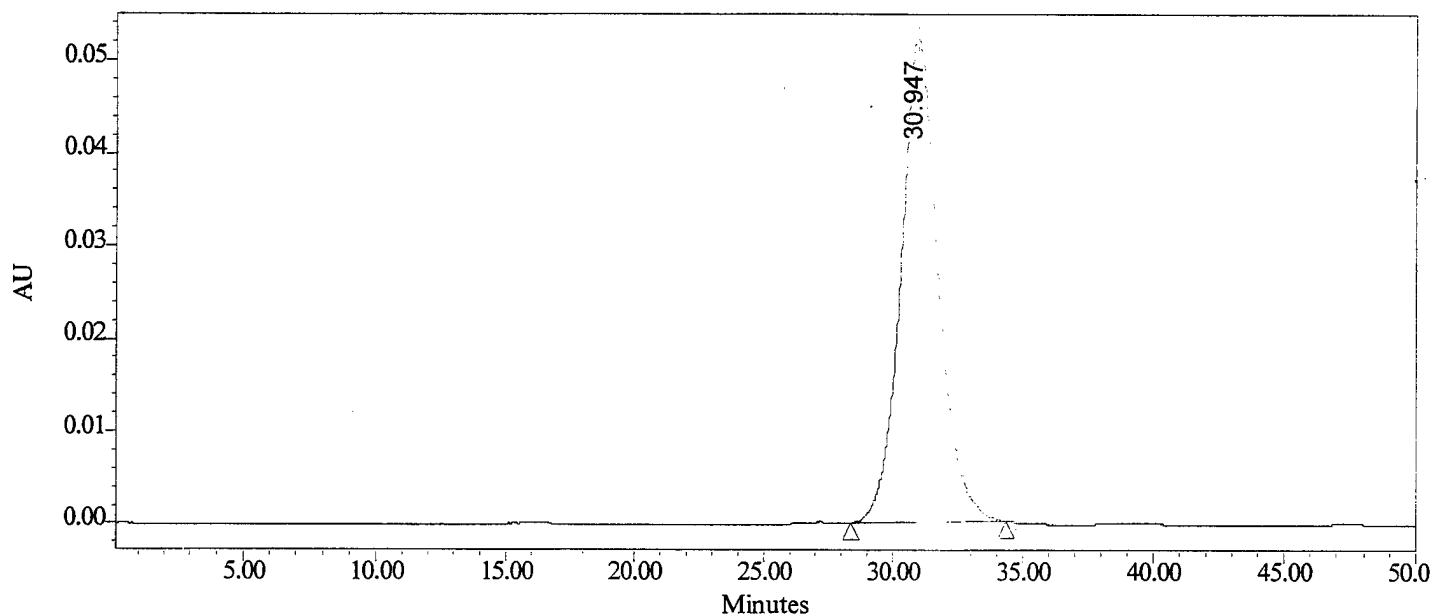
Figure 7. Fab Lot B14394, 394-19-21

Table 8. Fc Lot B27070, 394-20-21

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High molecular weight components	-	1.25	15.67
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
Fc	-	98.75	30.09

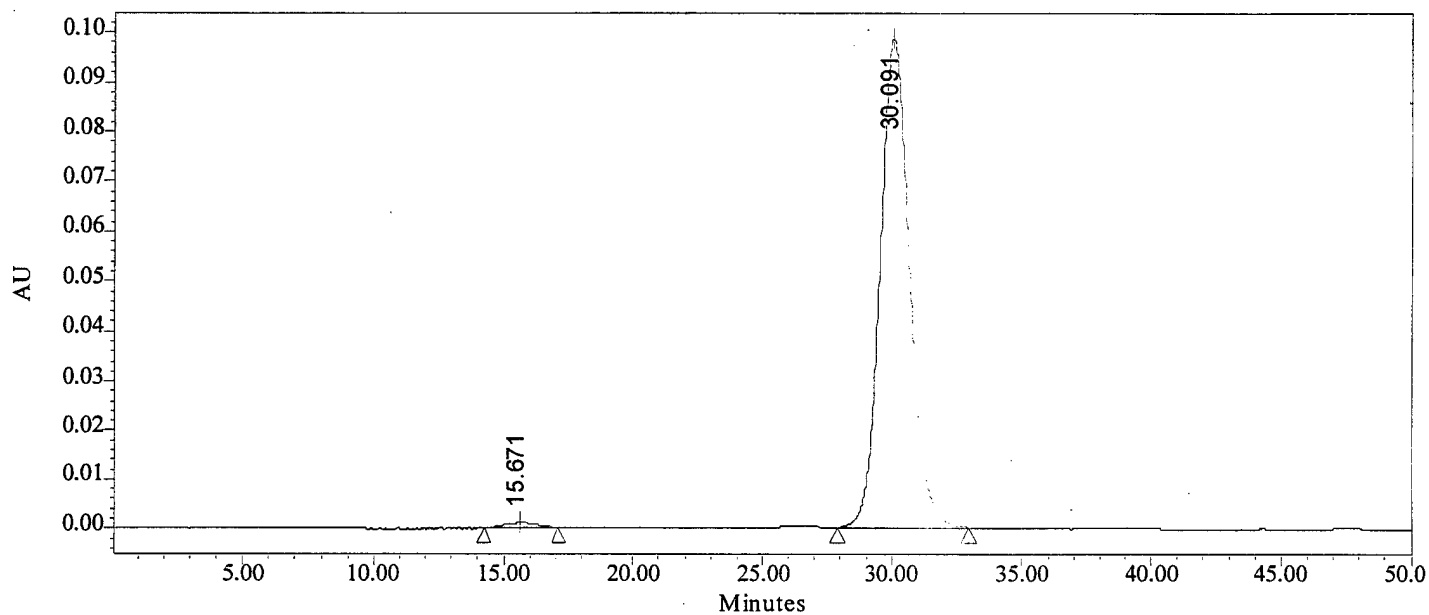
Figure 6. Fc Lot B27070, 394-20-21

Table 9. F(ab')₂ Lot B22818, 394-21-04

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	100.00	1.12	16.29
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
F(ab') ₂	-	98.25	26.57
Low molecular weight components	-	0.62	33.15

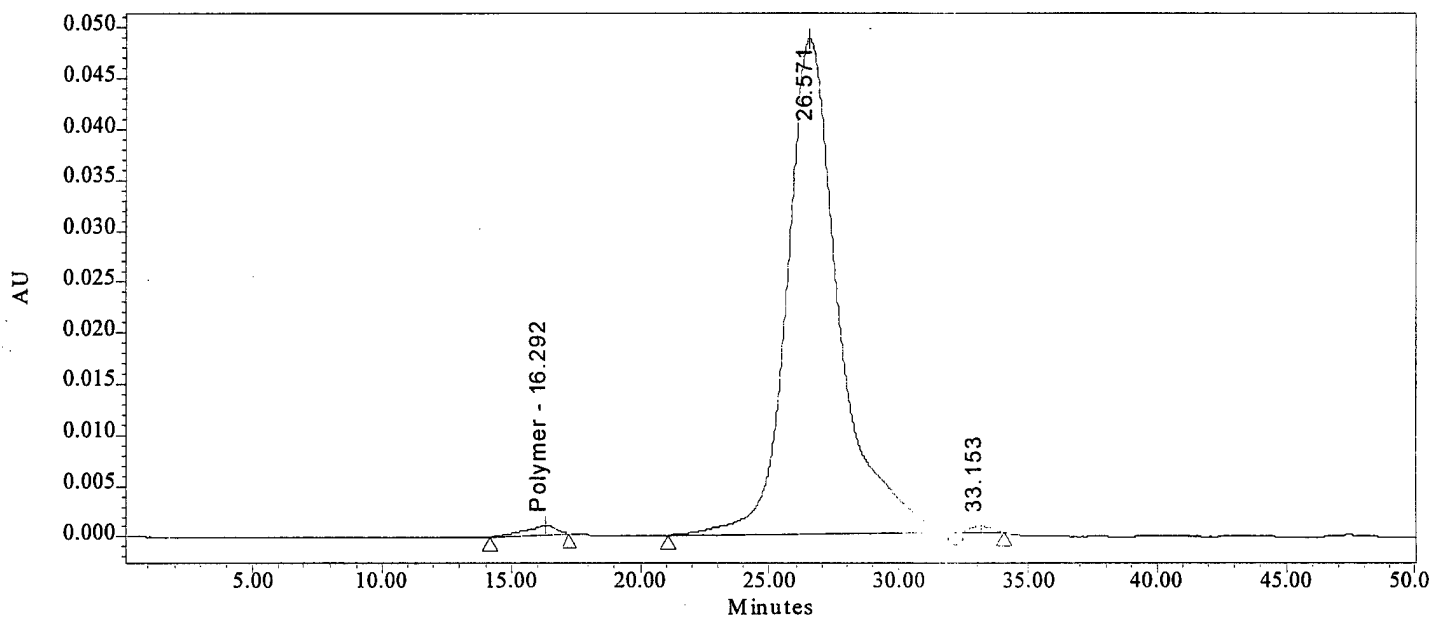
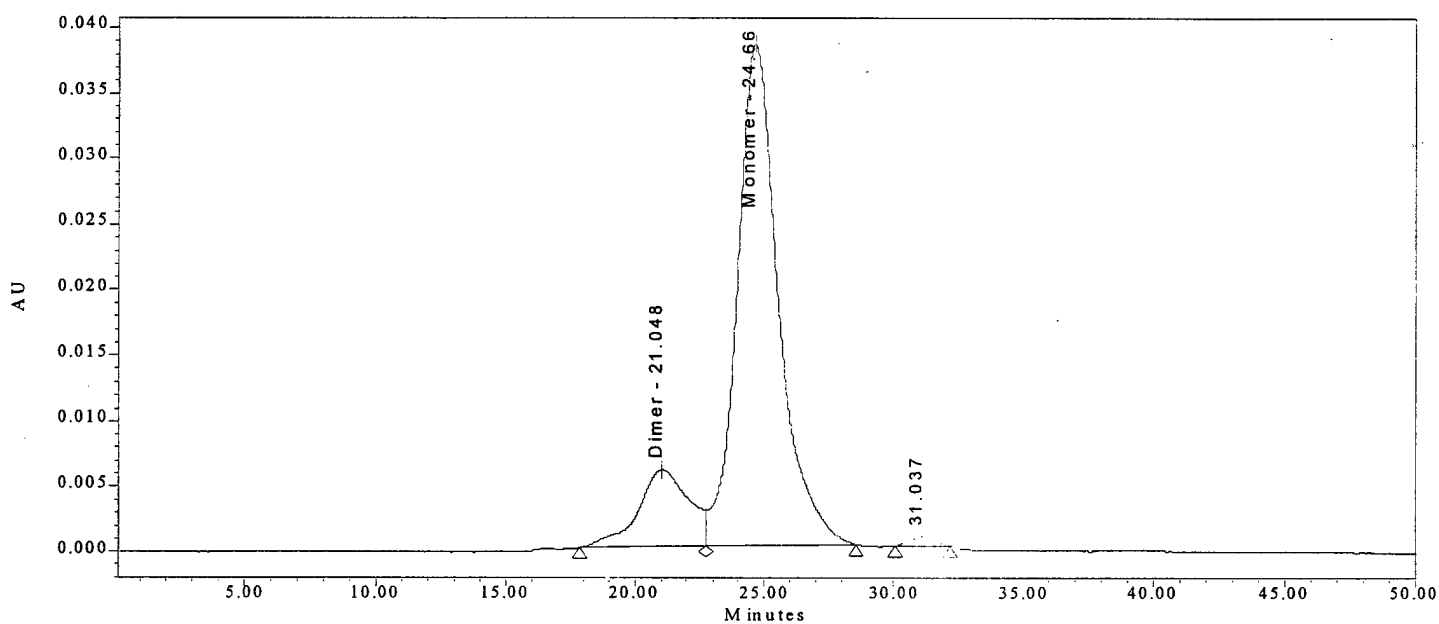
Figure 9. F(ab')₂ Lot B22818, 394-21-04

Table 10. IgG 394-20-06 2 mg/mL Lot B23973 frozen aliquot

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	15.76	15.61	21.05
IgG,Monomer	84.24	83.42	24.66
Low molecular weight components	-	0.97	31.04

Figure 10. IgG 394-20-06 2 mg/mL Lot B23973 frozen aliquot

Date December 8, 1999, 1999

To File

From Jon Kohne

JMK 12/08/99

Subject G1555-53A FPLC Analyses performed on
December 7, 1999

The data package for Task 53 FPLC analyses performed on December 7, 1999 consists of a ten page report addressed to Robert Hunt, and FPLC data from December 7, 1999-27 pages.

QC'd by:

Ted Miller 12-8-99

Reviewed by:

Tim Hayes 12-8-99

INTERNAL CHEMISTRY REPORT

from

Chemistry

on

Task 53

Project Number G155553A

to

Robert Hunt

December 8, 1999

by

Jon Kohne

Timothy Hayes

BATTELLE

Medical Research and Evaluation Facility

505 King Avenue, Building JM-3

Columbus, Ohio 43201-2693

Introduction

The chemistry group at the Medical Research and Evaluation Facility (MREF) was asked to evaluate the relative composition of components in antitoxin preparations. The analytical method was based on that published in: Botulism Immune Globulin (Human), Pentavalent, Lot 1A, 1B and 2A: Storage and Stability Report, The Salk Institute, July, 1996.

Experimental

The samples of lyophilized gammaglobulin of CIG were analyzed by modified FPLC (Fast Protein Liquid Chromatography) using a Waters Alliance Liquid Chromatograph equipped with a Waters 996 photodiode array detector (scan 200-600 nm, quantitate 280 nm). The separation was accomplished using a Superdex 200 HR column (Supelco, Bellefonte, PA, part number 17-1088-01) with 50 mM sodium phosphate buffer (pH 7) mobile phase. Known standards of antibody fragments IgG, Fab, F(ab')₂, and Fc (Calbiochem, La Jolla, CA) were chromatographed for comparison of retention times. The F(ab')₂ was received in a solution with the concentration listed as 2.14 mg/mL protein. The IgG, Fab and Fc were received as dry powders. The IgG and Fab were diluted to a concentration of 2 mg/mL with mobile phase. The Fc was diluted to a concentration of 2.5 mg/mL with mobile phase. A detailed description of the analytical procedure is contained in Chemistry Method No. 14, February 13, 1998.

Standards

1. Human Immunoglobulin G, Plasma, IgG, Calbiochem, catalog number 401114, lot number B27643, sample number 394-24-16.
2. Human Immunoglobulin G, Fab Fragment, Plasma, Calbiochem, catalog number 401116, lot number B14394, sample number 394-25-03.
3. Human Immunoglobulin G, F(ab')₂ Fragment, Plasma, Calbiochem, catalog number 401103, lot number B27607, sample number 394-25-16.
4. Human Immunoglobulin G, Fc Fragment, Plasma, Calbiochem, catalog number 401104, lot number B29864, sample number 394-26-03.

Samples

The following samples were received for analysis. Approximately 2 mg of powder was weighed into a 1.0-mL volumetric flask and diluted to volume with mobile phase. :

1. Lyophilized gammaglobulin – Test #1, Pbig A, Box #1, Lot D1103.
2. Lyophilized gammaglobulin – Test #2, Pbig A, Box #4, Lot D1103.
3. Lyophilized gammaglobulin – Test #3, Pbig A, Box #6, Lot D1103.

Results

Tables 1-7 contain a list of the retention times and the relative percent area of peaks in the chromatograms of the IgG, Fab, F(ab')₂, Fc and samples. Chromatograms 1-7 are included after each corresponding table. The samples and standards were run in duplicate to verify reproducibility, however, the chromatograms and tables contain data from only the first injection. The %RSD was less than 3.0% for the peak areas of the sample and standards.

Discussion

The samples contained peaks at the same retention time of the IgG monomer. The average area percent of this peak for the samples was 100 percent.

Table 1. IgG Std. 394-24-16 2 mg/mL Lot# B27643

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	3.30	3.30	20.77
IgG,Monomer	96.70	96.70	24.36

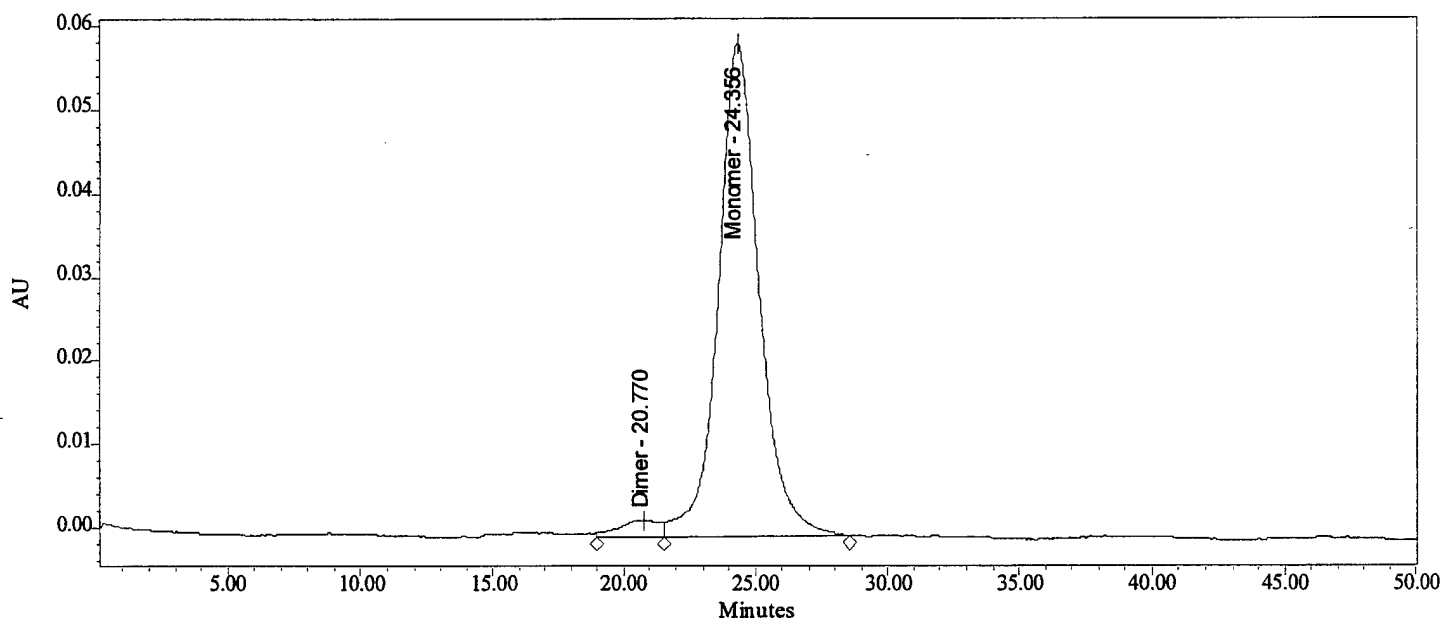
Figure 1. IgG Std. 394-24-16 2 mg/mL Lot# B27643

Table 2. Sample 1 – 394-26-19, Test# 1, Pbig A, Box#1, IgG Lot D1103

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	-	-	21.14
IgG,Monomer	100.00	100.00	24.35

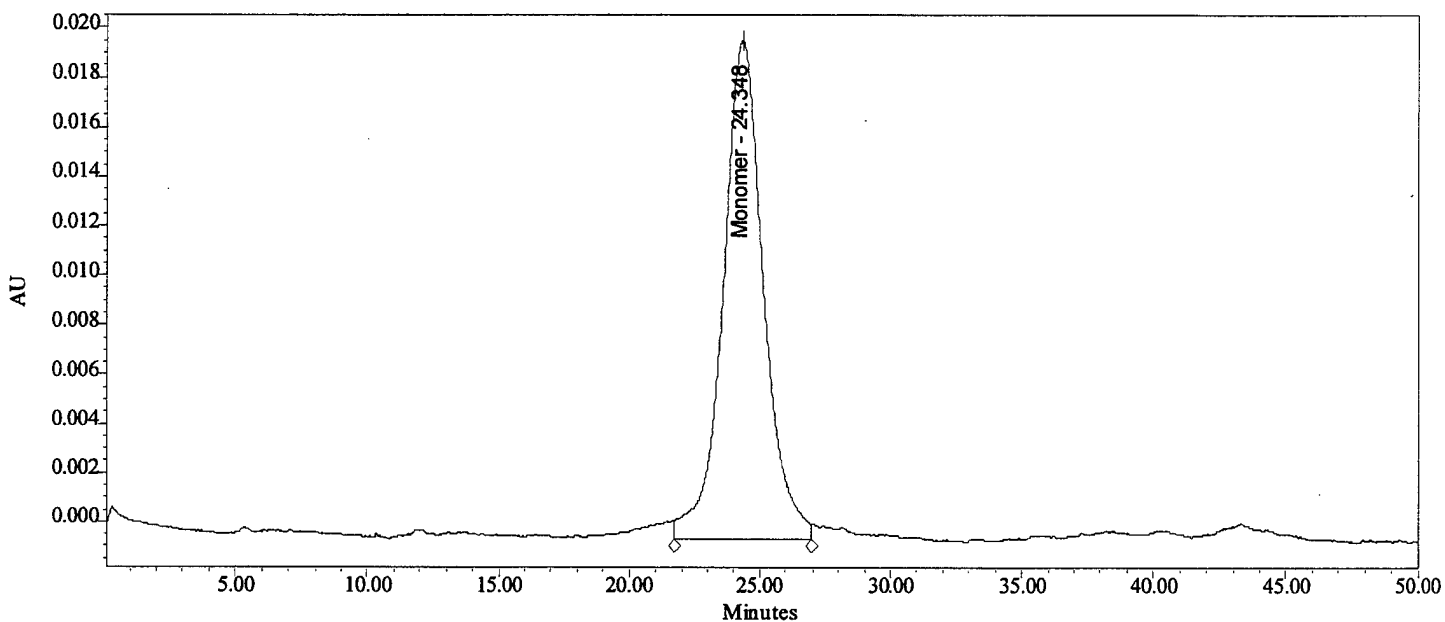
Figure 2. Sample 1 - 394-26-19, Test# 1, Pbig A, Box#1, IgG Lot D1103

Table 3. Sample 2- 394-26-25 Test# 2, Pbig A, Box# 4, IgG Lot#D1103

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	-	-	21.14
IgG,Monomer	100.00	100.00	24.34

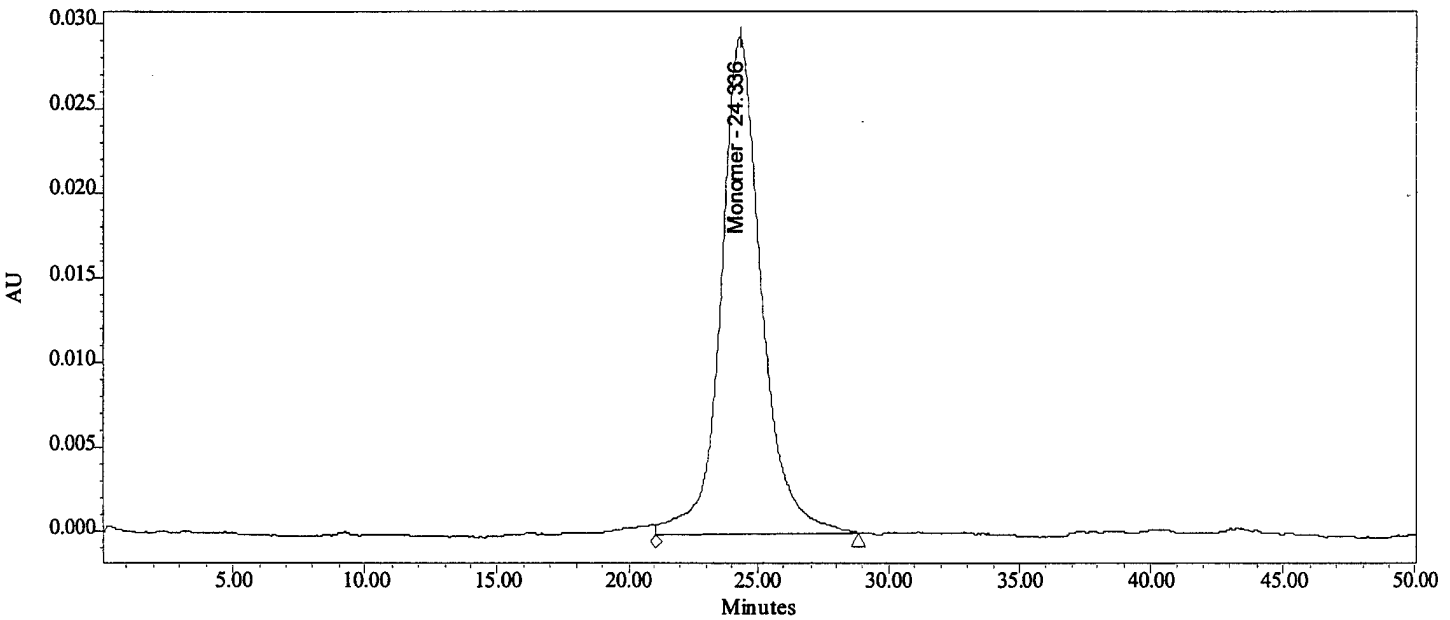
Figure 3. Sample 2- 394-26-25 Test# 2, Pbig A, Box# 4, IgG Lot#D1103

Table 4. Sample 3- 394-26-31 Test# 3, Pbig A, Box# 6, IgG Lot#D1103

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	-	-	21.14
IgG,Monomer	100.00	100.00	24.34

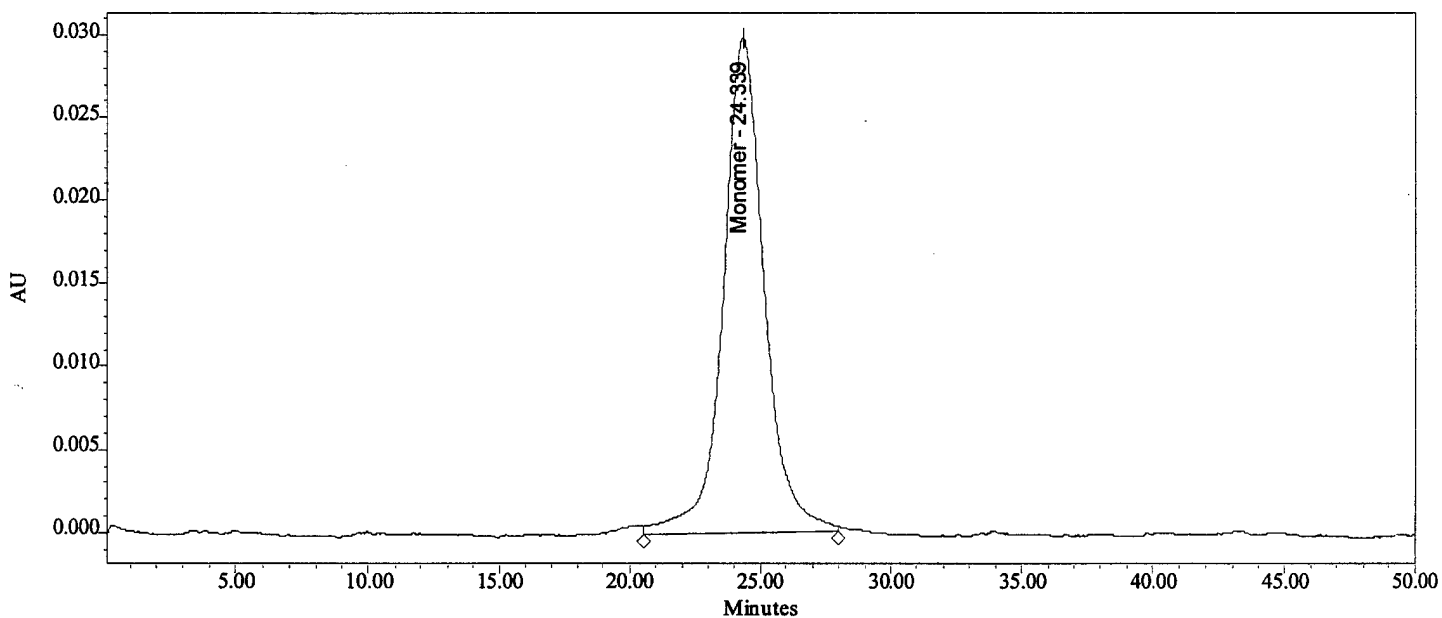
Figure 4. Sample 3- 394-26-31 Test# 3, Pbig A, Box# 6, IgG Lot#D1103

Table 5. IgG, FAB Std. 394-25-03 2 mg/mL Lot# B14394

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
FAB	-	100.00	30.68

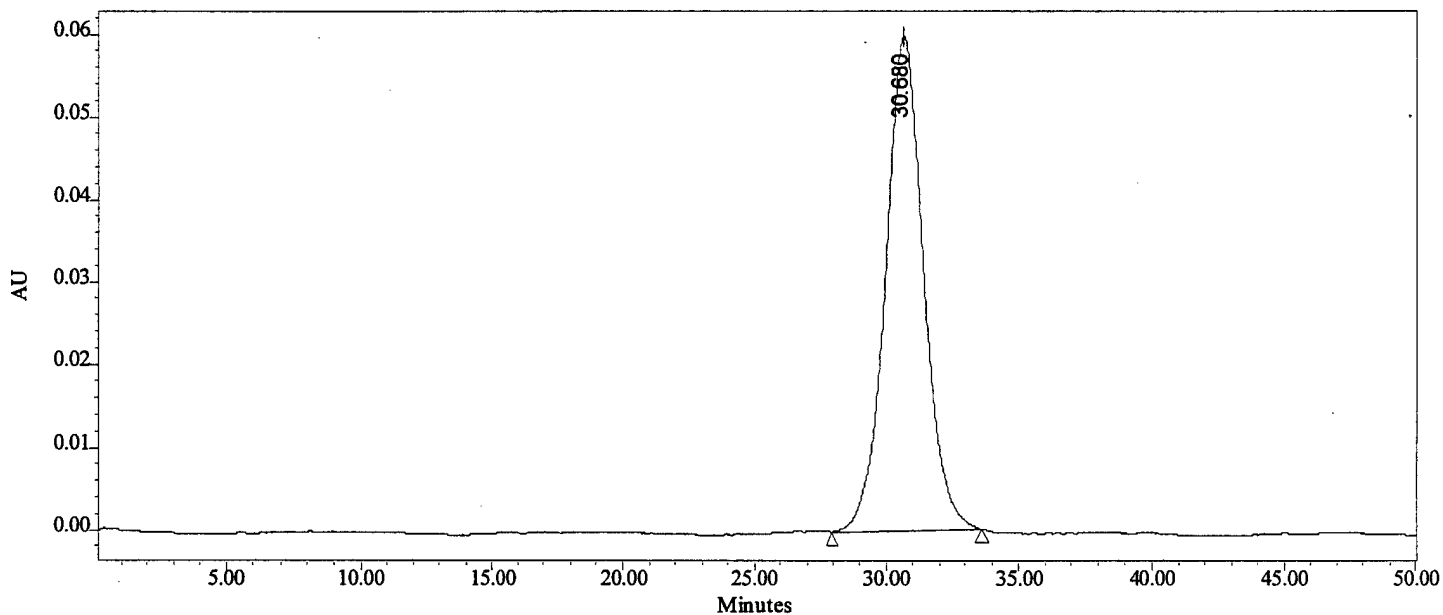
Figure 5. IgG, FAB Std. 394-25-03 2 mg/mL Lot# B14394

Table 6. IgG, F(ab')₂ Std. 394-25-16 2.14 mg/mL Lot# B27607

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
F(ab') ₂	-	100.00	26.31

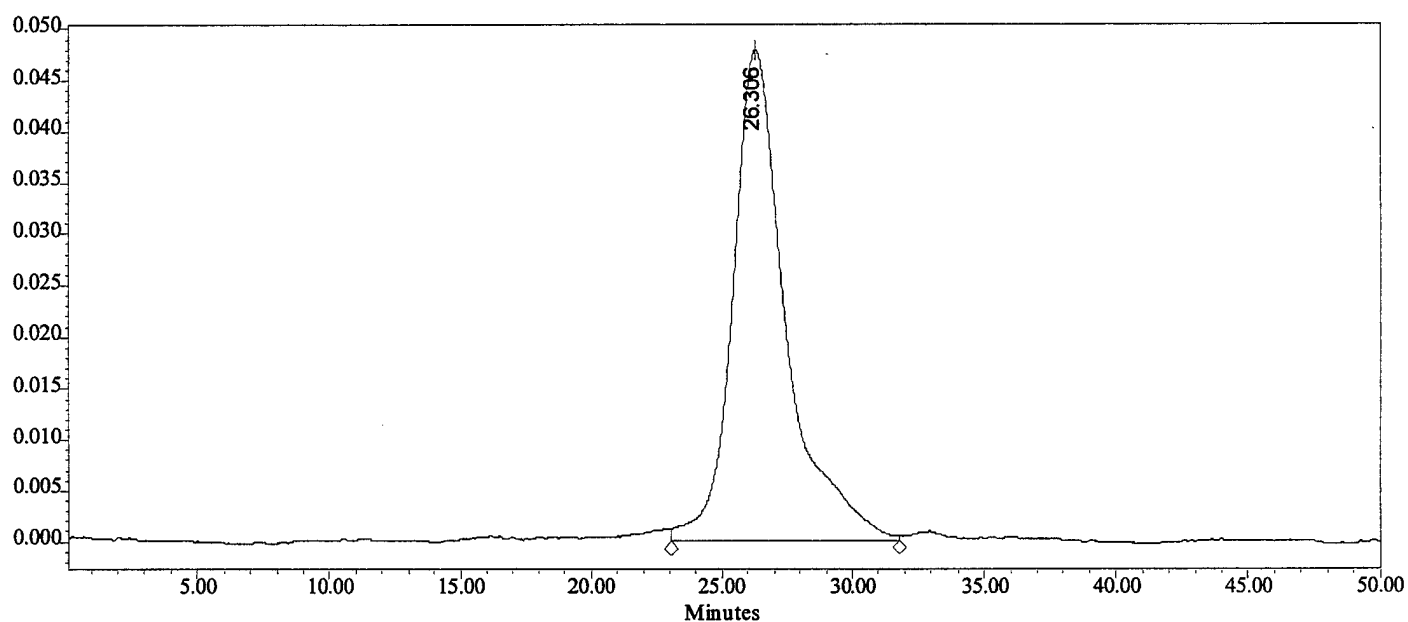
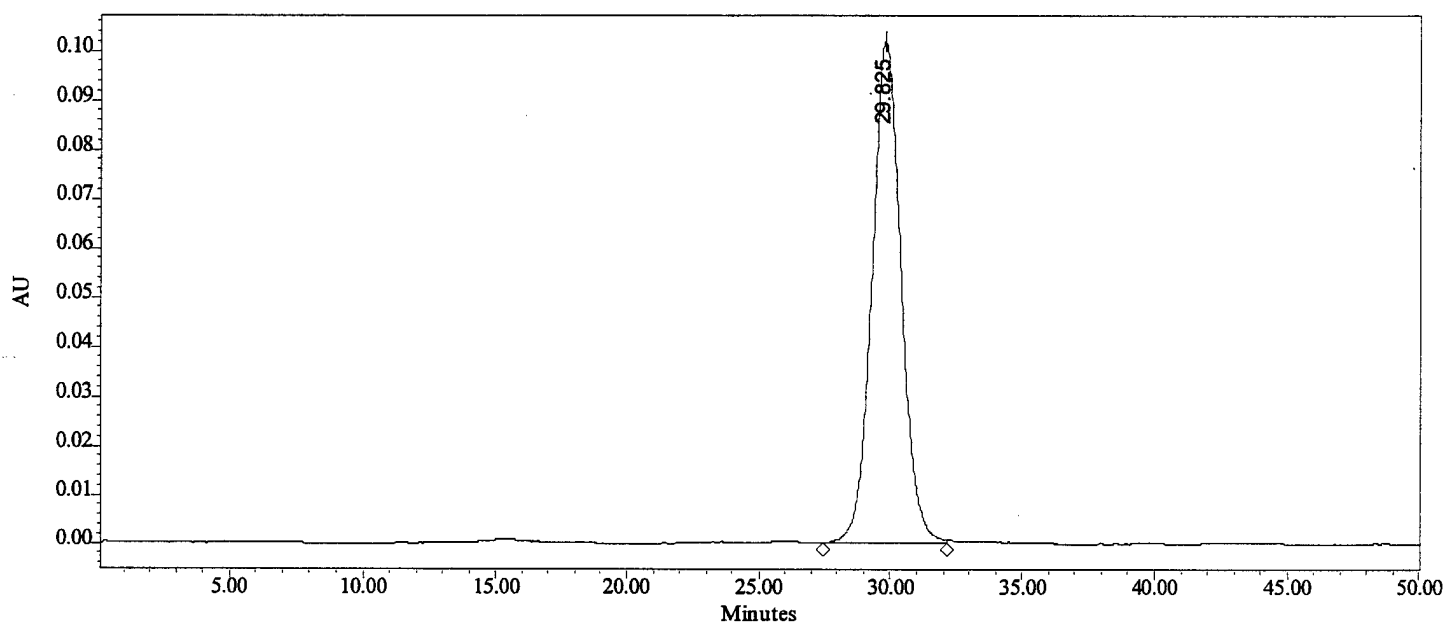
Figure 6. IgG, F(ab')₂ Std. 394-25-16 2.14 mg/mL Lot# B27607

Table 7. IgG, Fc Std. 394-26-03 2.5 mg/mL Lot# B29864

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
Fc	-	100.00	29.83

Figure 7. IgG, Fc Std. 394-26-03 2.5 mg/mL Lot# B29864

Date 06-January-2000

To File

From Jon Kohne *JK*

Subject G1555-53A FPLC Analyses performed on 05-
January-2000

The data package for Task 53 FPLC analyses performed on 05-January-2000 consists of a ten page report addressed to Robert Hunt, and FPLC data from 05-January-2000-44 pages.

QC'd by:

David Nitz 1-7-00

Reviewed by:

Tim Hays 1-12-00

INTERNAL CHEMISTRY REPORT

from

Chemistry

on

Task 53

Project Number G155553A

to

Robert Hunt

06-January-2000

by

Jon Kohne

Timothy Hayes

BATTELLE

Medical Research and Evaluation Facility

505 King Avenue, Building JM-3

Columbus, Ohio 43201-2693

Introduction

The chemistry group at the Medical Research and Evaluation Facility (MREF) was asked to evaluate the relative composition of components in antibody preparations. The analytical method was based on that published in: Botulism Immune Globulin (Human), Pentavalent, Lot 1A, 1B and 2A: Storage and Stability Report, The Salk Institute, July, 1996.

Experimental

The samples of lyophilized gammaglobulin of PBig-B were analyzed by modified FPLC (Fast Protein Liquid Chromatography) using a Waters Alliance Liquid Chromatograph equipped with a Waters 996 photodiode array detector (scan 200-600 nm, quantitate 280 nm). The separation was accomplished using a Superdex 200 HR column (Supelco, Bellefonte, PA, part number 17-1088-01) with 50 mM sodium phosphate buffer (pH 7) mobile phase. Known standards of antibody fragments IgG, Fab, F(ab')₂, and Fc (Calbiochem, La Jolla, CA) were chromatographed for comparison of retention times. The F(ab')₂ was received in a solution with the concentration listed as 2.14 mg/mL protein. The IgG, Fab and Fc were received as dry powders. The IgG and Fab were diluted to a nominal concentration of 2 mg/mL with mobile phase. The Fc was diluted to a nominal concentration of 2.5 mg/mL with mobile phase. A detailed description of the analytical procedure is contained in Chemistry Method No. 14, February 13, 1998.

Standards

1. Human Immunoglobulin G, Plasma, IgG, Calbiochem, catalog number 401114, lot number B27643, sample number 394-24-16.
2. Human Immunoglobulin G, Fab Fragment, Plasma, Calbiochem, catalog number 401116, lot number B14394, sample number 394-25-03.
3. Human Immunoglobulin G, F(ab')₂ Fragment, Plasma, Calbiochem, catalog number 401103, lot number B27607, sample number 394-25-16.
4. Human Immunoglobulin G, Fc Fragment, Plasma, Calbiochem, catalog number 401104, lot number B29864, sample number 394-26-03.

Samples

The following samples were received for analysis. The samples were already diluted to a nominal concentration of 50 mg/mL in sterile water. They were further diluted to 2.0 mg/mL by placing 200 L into a 5.0-mL volumetric flask and diluting to volume with mobile phase. :

1. Lyophilized gammaglobulin – PBig B, Vial #1, Lot D1105.
2. Lyophilized gammaglobulin – PBig B, Vial #2, Lot D1105.
3. Lyophilized gammaglobulin – PBig B, Vial #3, Lot D1105.

Results

Tables 1-7 contain a list of the retention times and the relative percent area of peaks in the chromatograms of the IgG, Fab, F(ab')₂, Fc and samples. Chromatograms 1-7 are included after each corresponding table. The samples and standards were run in duplicate to verify reproducibility, however, the chromatograms and tables contain data from only the first injection. The %RSD was less than 5.0% for the peak areas of the samples and standards.

Discussion

The samples contained peaks at the same retention time of the IgG monomer. The average area percent of this peak for the samples was 97.4 percent.

Table 1. IgG Std. 394-27-16 2 mg/mL Lot# B27643

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	3.80	3.80	20.46
IgG,Monomer	96.20	96.20	24.06

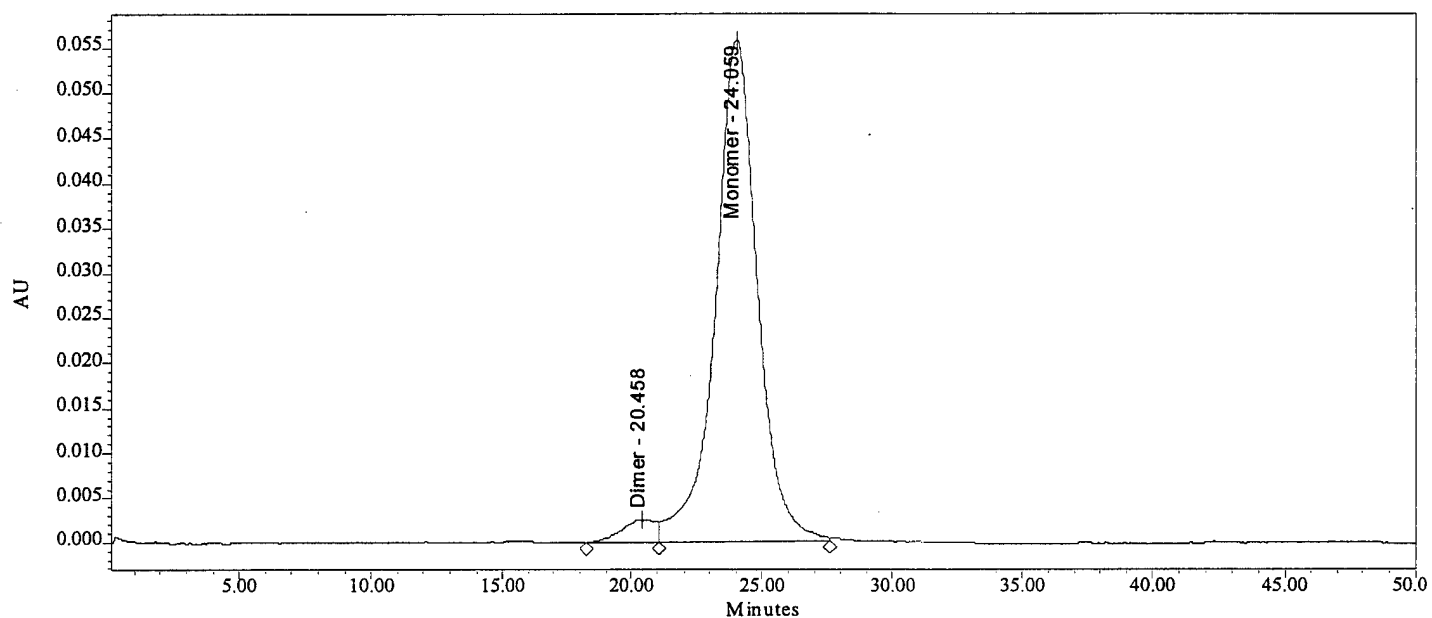
Figure 1. IgG Std. 394-27-16 2 mg/mL Lot# B27643

Table 2. Sample 1 – 394-30-04, PBig B, Vial #1, Lot D1105

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.37	0.37	16.08
IgG,Dimer	1.85	1.83	20.60
IgG,Monomer	97.78	97.12	24.03
Low Molecular Weight Components	-	0.18	38.29
Low Molecular Weight Components	-	0.50	40.20

Figure 2. Sample 1 – 394-30-04, PBig B, Vial #1, Lot D1105

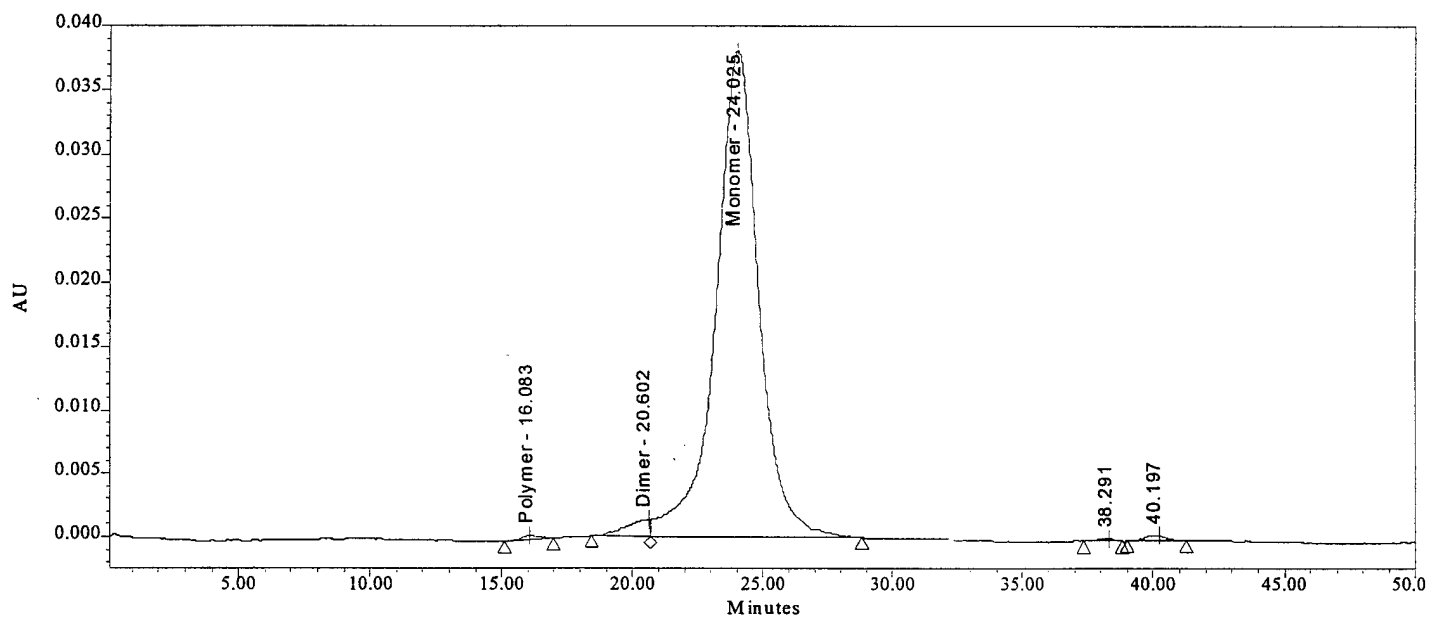


Table 3. Sample 2 – 394-30-08, PBig B, Vial #2, Lot D1105

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.31	0.31	16.06
IgG,Dimer	2.27	2.26	21.01
IgG,Monomer	97.42	97.09	24.03
Low Molecular Weight Components	-	0.34	40.19

Figure 3. Sample 2 – 394-30-08, PBig B, Vial #2, Lot D1105

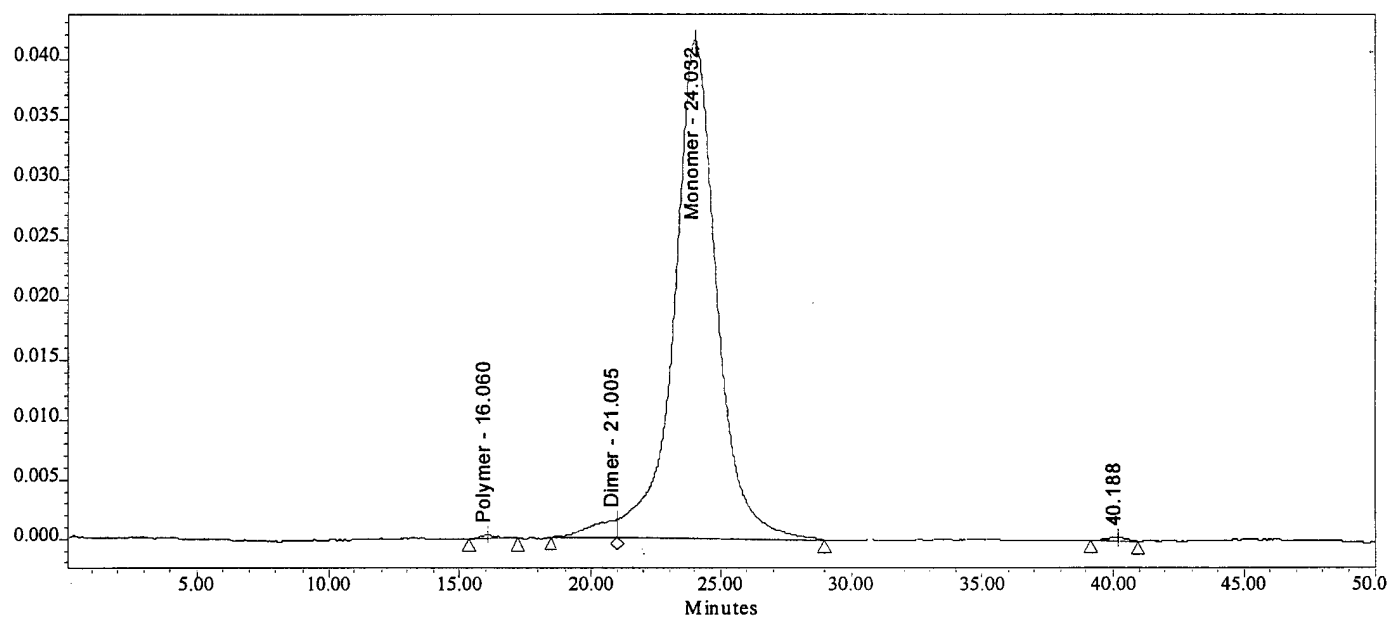


Table 4. Sample 3 – 394-30-12, PBig B, Vial #3, Lot D1105

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.23	0.23	16.05
IgG,Dimer	2.97	2.95	20.86
IgG,Monomer	97.00	96.39	24.04
Low Molecular Weight Components	-	0.42	40.10

Figure 4. Sample 3 – 394-30-12, PBig B, Vial #3, Lot D1105

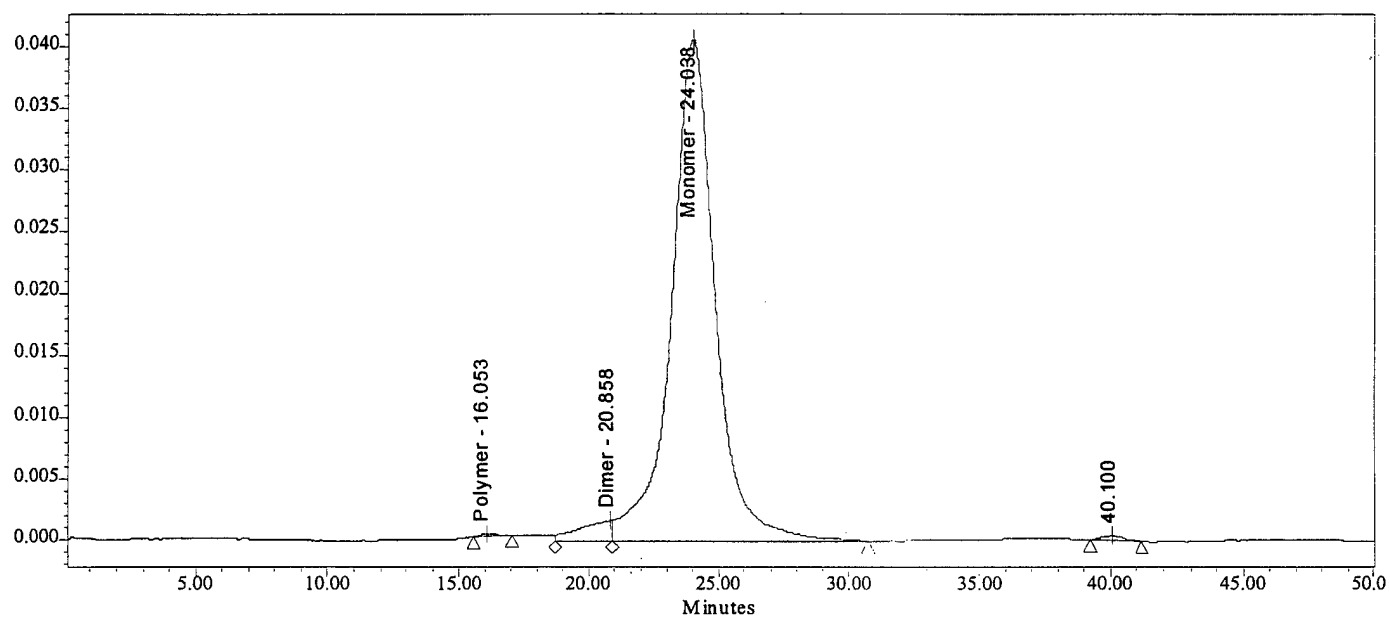


Table 5. IgG, Fab Std. 394-28-04 2 mg/mL Lot# B14394

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
Fab	-	100.00	30.39

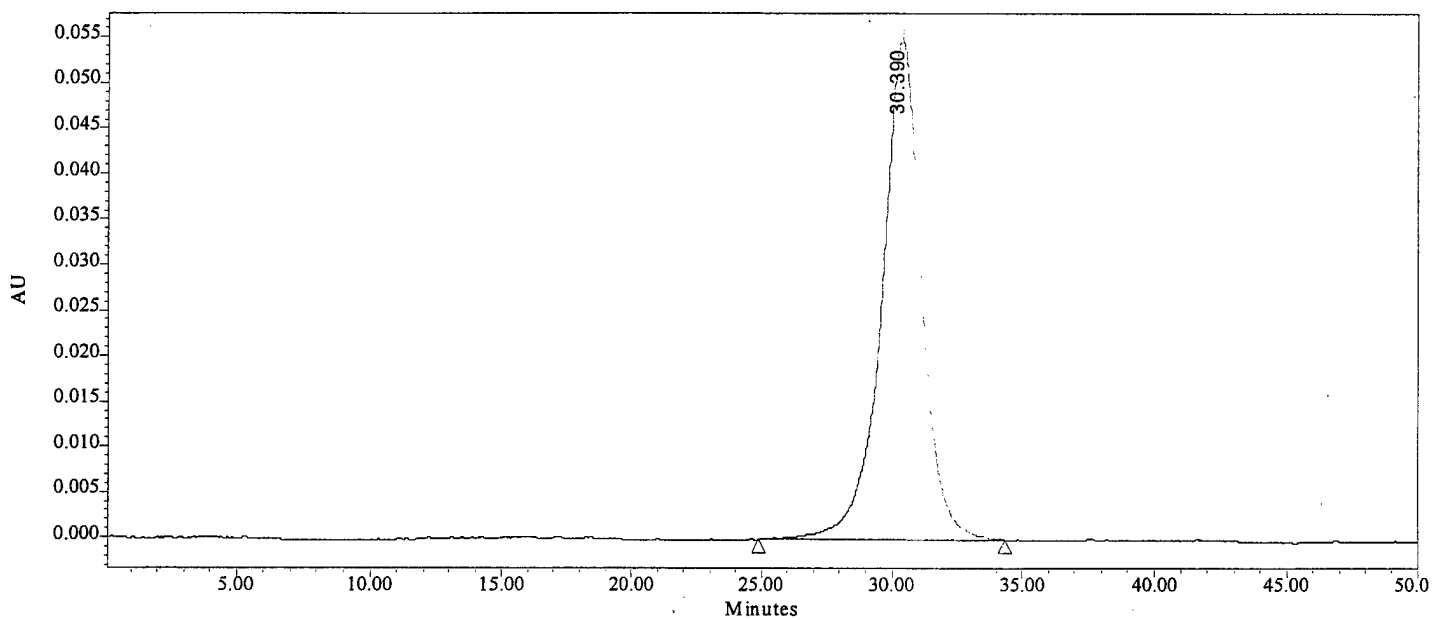
Figure 5. IgG, Fab Std. 394-28-04 2 mg/mL Lot# B14394

Table 6. IgG, F(ab')₂ Std. 394-28-18 2.14 mg/mL Lot# B27607

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Components	-	1.15	15.52
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
F(ab') ₂	-	97.61	25.99
Low Molecular Weight Components	-	1.24	32.51

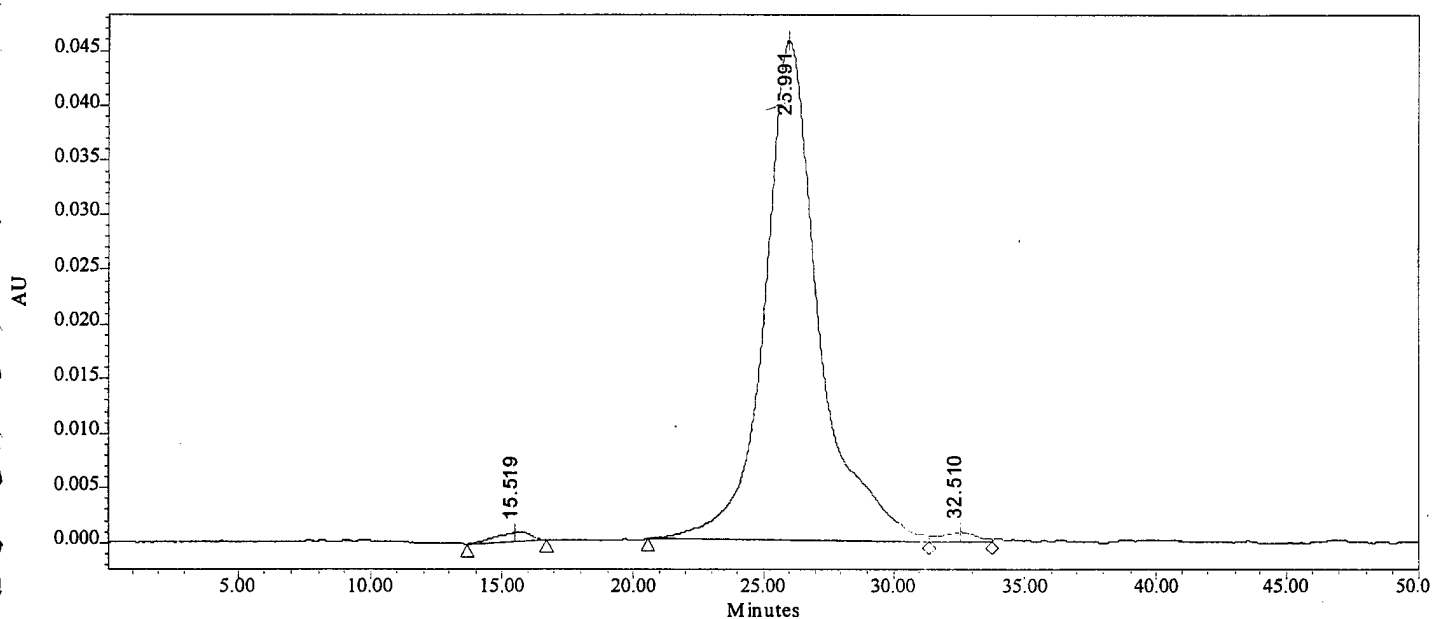
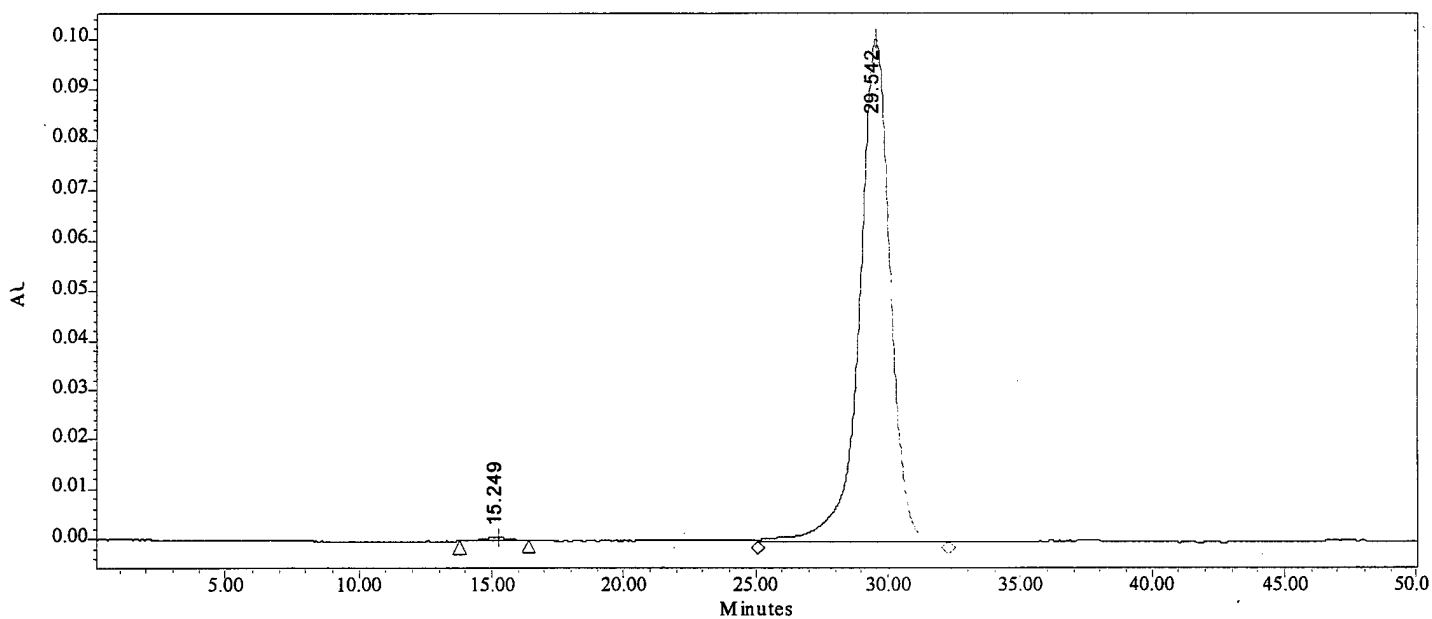
Figure 6. IgG, F(ab')₂ Std. 394-28-18 2.14 mg/mL Lot# B27607

Table 7. IgG, Fc Std. 394-29-03 2.5 mg/mL Lot# B29864

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Components	-	0.62	15.25
IgG, Polymer	-	-	-
IgG, Dimer	-	-	-
IgG, Monomer	-	-	-
Fc	-	99.38	29.54

Figure 7. IgG, Fc Std. 394-29-03 2.5 mg/mL Lot# B29864

Date 25-February-2000

To File

From Jon Kohne

JWK 02/25/00

Subject G1555-53A FPLC Analyses performed on 24-
February-2000

The data package for Task 53 FPLC analyses performed on 24-February-2000 consists of an eight page report addressed to Robert Hunt, and FPLC data from 24- February -2000-30 pages.

QC'd by:

Jon Kohne 4-13-00

Reviewed by:

Tim Hayes 4/13/00

INTERNAL CHEMISTRY REPORT

from

Chemistry

on

Task 53

Project Number G155553A

to

Robert Hunt

25-February-2000

by

Jon Kohne

Timothy Hayes

BATTELLE

Medical Research and Evaluation Facility

505 King Avenue, Building JM-3

Columbus, Ohio 43201-2693

Introduction

The chemistry group at the Medical Research and Evaluation Facility (MREF) was asked to evaluate the relative composition of components in antibody preparations. The analytical method was based on that published in: Botulism Immune Globulin (Human), Pentavalent, Lot 1A, 1B and 2A: Storage and Stability Report, The Salk Institute, July, 1996.

Experimental

The samples of lyophilized gammaglobulin of BBig-A were analyzed by modified FPLC (Fast Protein Liquid Chromatography) using a Waters Alliance Liquid Chromatograph equipped with a Waters 996 photodiode array detector (scan 200-600 nm, quantitate 280 nm). The separation was accomplished using a Superdex 200 HR column (Supelco, Bellefonte, PA, part number 17-1088-01) with 50 mM sodium phosphate buffer (pH 7) mobile phase. Known standards of antibody fragments IgG, Fab, F(ab')₂, and Fc (Calbiochem, La Jolla, CA) were chromatographed for comparison of retention times. The F(ab')₂ was received in a solution with the concentration listed as 2.14 mg/mL protein. The IgG, Fab and Fc were received as dry powders. The IgG and Fab were diluted to a nominal concentration of 2 mg/mL with mobile phase. The Fc was diluted to a nominal concentration of 2.5 mg/mL with mobile phase. A detailed description of the analytical procedure is contained in Chemistry Method No. 14, February 13, 1998.

Standards

1. Human Immunoglobulin G, Plasma, IgG, Calbiochem, catalog number 401114, lot number B27643, sample number 394-24-16.
2. Human Immunoglobulin G, Fab Fragment, Plasma, Calbiochem, catalog number 401116, lot number B14394, sample number 394-25-03.
3. Human Immunoglobulin G, F(ab')₂ Fragment, Plasma, Calbiochem, catalog number 401103, lot number B27607, sample number 394-25-16.
4. Human Immunoglobulin G, Fc Fragment, Plasma, Calbiochem, catalog number 401104, lot number B29864, sample number 394-26-03.

Samples

The following sample was received for analysis. Approximately 4 mg of sample was weighed into a 2.0-mL volumetric flask and diluted to a nominal concentration of 2.0 mg/mL in mobile phase.

1. Lyophilized gammaglobulin – BBig A, Bottle #1, Lot D1116.

Results

Tables 1-5 contain a list of the retention times and the relative percent area of peaks in the chromatograms of the IgG, Fab, F(ab')₂, Fc and samples. Chromatograms 1-5 are included after each corresponding table. The samples and standards were run in duplicate to verify reproducibility, however, the chromatograms and tables contain data from only the first injection. The %RSD between injections was less than 3.0% for the peak areas of the sample and standards.

Discussion

The sample contained peaks at the same retention time of the IgG monomer. The average area percent of this peak for the sample was 90.5 percent.

Table 1. IgG Std. 394-31-16 2 mg/mL Lot# B27643

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	16.75
IgG,Dimer	3.97	3.97	20.45
IgG,Monomer	96.03	96.03	24.09

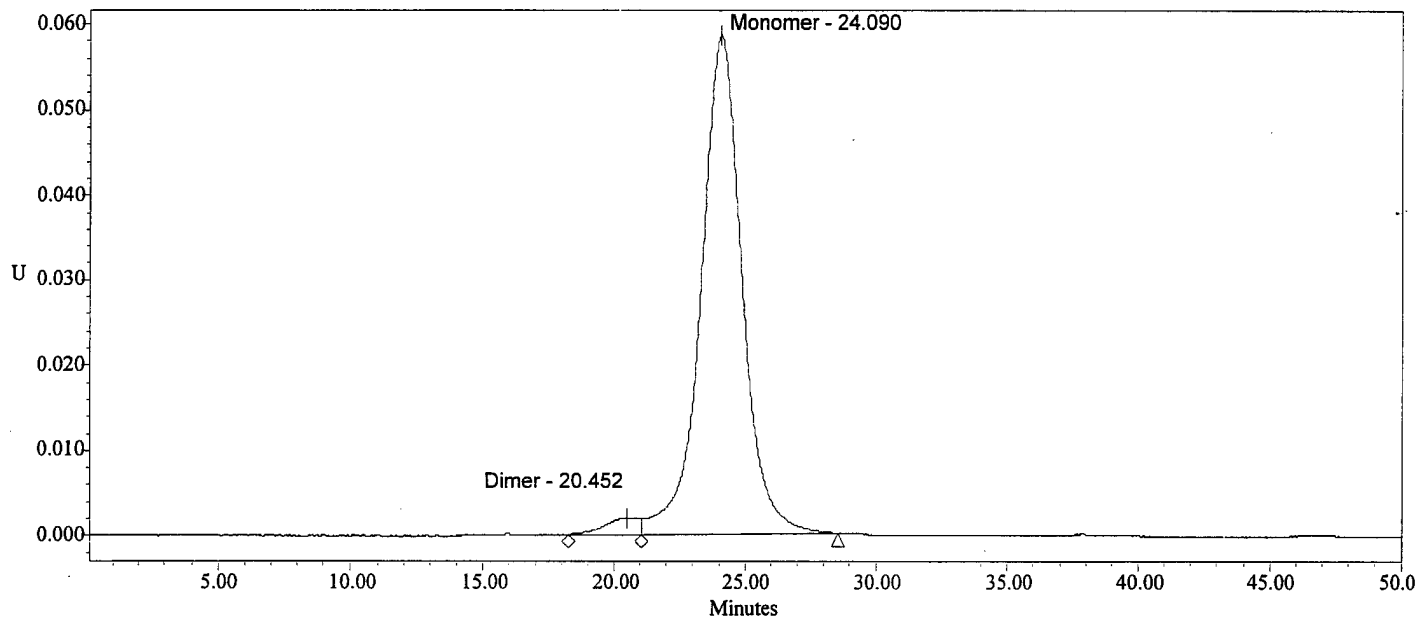
Figure 1. IgG Std. 394-31-16 2 mg/mL Lot# B27643

Table 2. Sample 1 – 394-33-21, BBig A, Bottle #1, Lot D1116

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	0.37	0.42	16.08
IgG,Dimer	1.85	2.63	21.43
IgG,Monomer	97.78	90.57	24.06
Low Molecular Weight Components	-	6.38	40.07

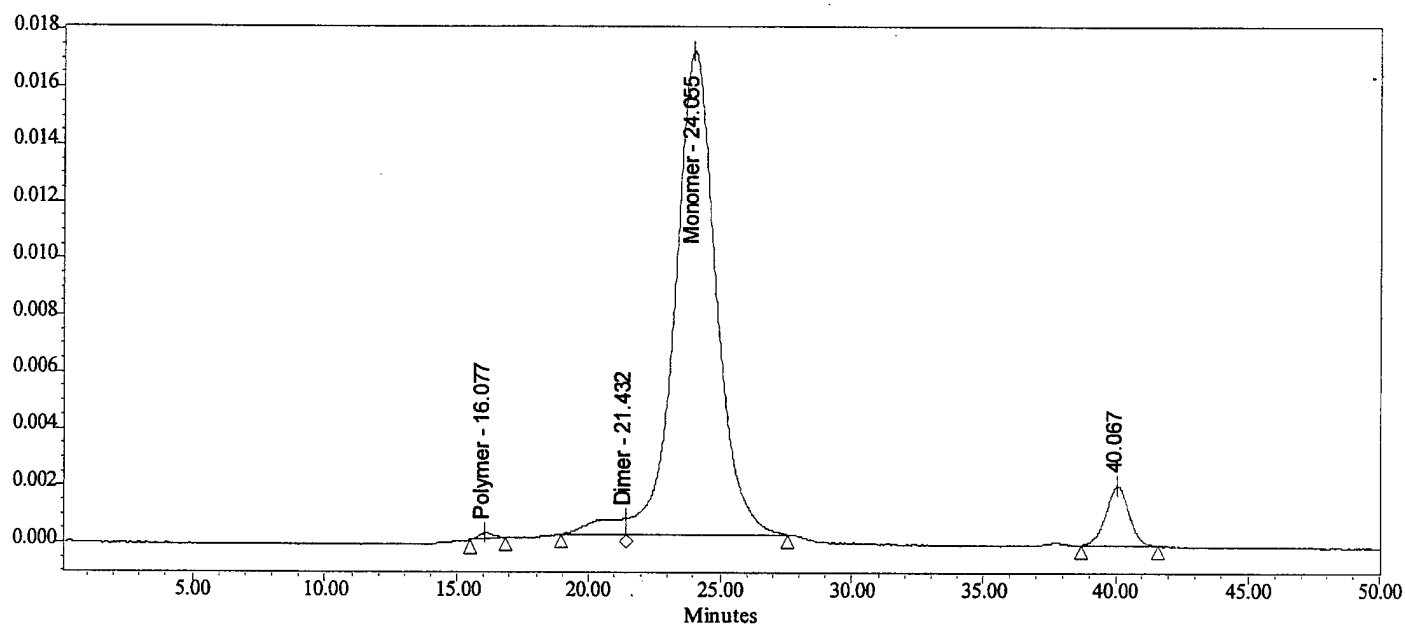
Figure 2. Sample 1 – 394-33-21, BBig A, Bottle #1, Lot D1116

Table 3. IgG, Fab Std. 394-32-05 2 mg/mL Lot# B14394

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
Fab	-	100.00	30.42

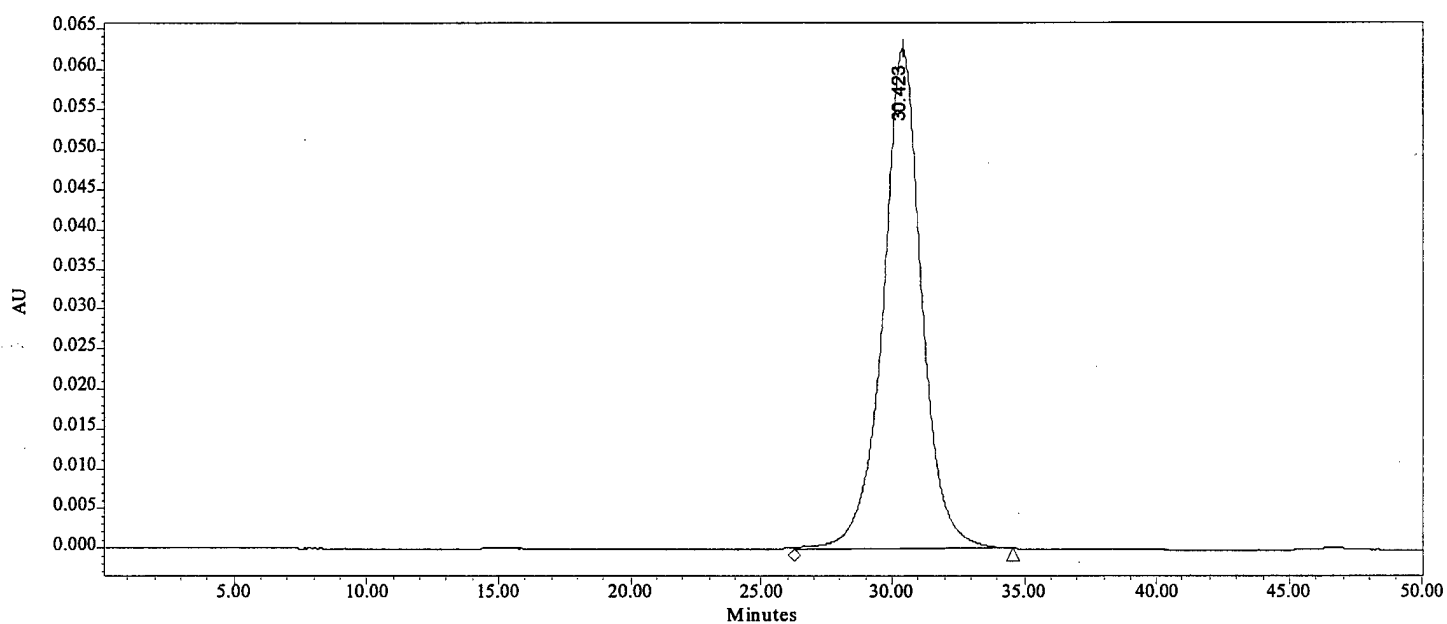
Figure 3. IgG, Fab Std. 394-32-05 2 mg/mL Lot# B14394

Table 4. IgG, F(ab')₂ Std. 394-32-17 2.14 mg/mL Lot# B27607

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Components	-	1.35	15.77
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
F(ab') ₂	-	97.69	26.05
Low Molecular Weight Components	-	0.97	32.57

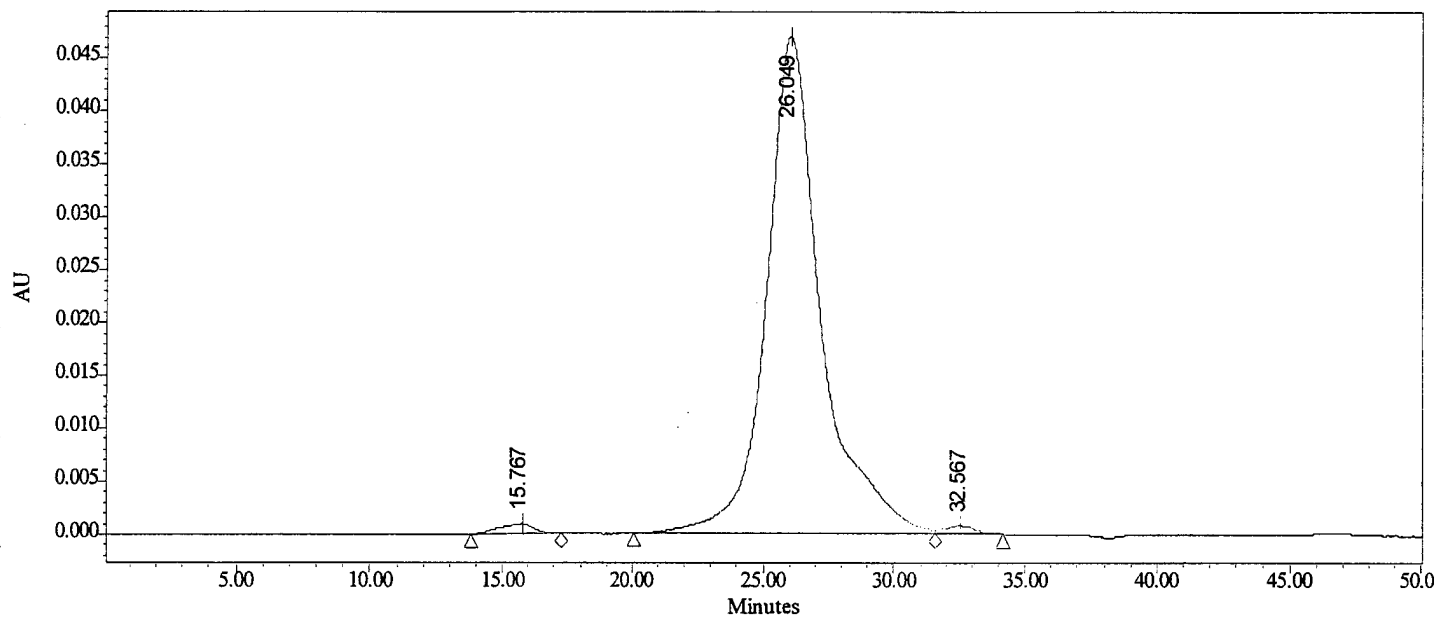
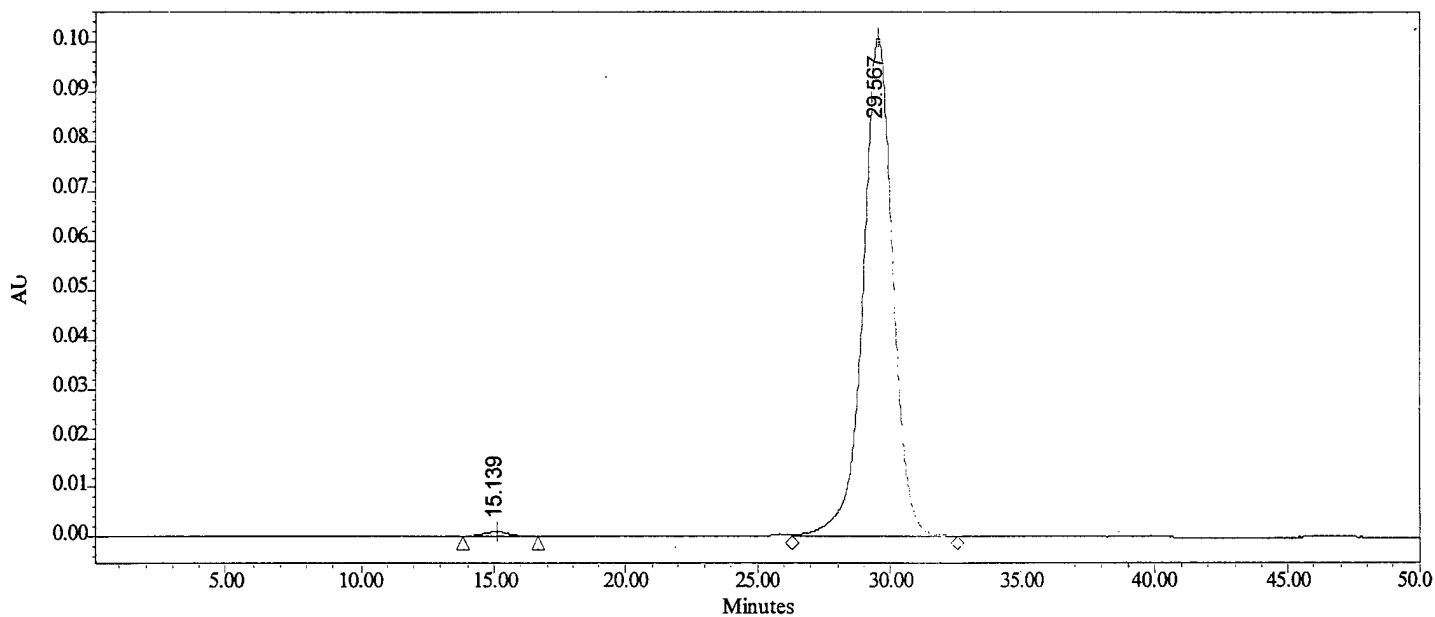
Figure 4. IgG, F(ab')₂ Std. 394-32-17 2.14 mg/mL Lot# B27607

Table 5. IgG, Fc Std. 394-33-05 2.5 mg/mL Lot# B29864

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Components	-	0.93	15.14
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
Fc	-	99.07	29.57

Figure 5. IgG, Fc Std. 394-33-05 2.5 mg/mL Lot# B29864

Date 20-April-2000

To File

From Jonathon Kohne *JKW 4/20/00*

Subject G1555-53A FPLC Analyses performed on 19-April-2000

The data package for Task 53 FPLC analyses performed on 19-April-2000 consists of an eight page report addressed to Robert Hunt, and FPLC data from 19-April -2000-32 pages.

QC'd by:

TLM 4-21-00

Reviewed by:

Tom Hoyer 4-21-00

INTERNAL CHEMISTRY REPORT

from

Chemistry

on

Task 53

Project Number G155553A

to

Robert Hunt

20-April-2000

by

Jonathon Kohne

Timothy Hayes

BATTELLE

Medical Research and Evaluation Facility

505 King Avenue, Building JM-3

Columbus, Ohio 43201-2693

Introduction

The chemistry group at the Medical Research and Evaluation Facility (MREF) was asked to evaluate the relative composition of components in antibody preparations. The analytical method was based on that published in: Botulism Immune Globulin (Human), Pentavalent, Lot 1A, 1B and 2A: Storage and Stability Report, The Salk Institute, July, 1996.

Experimental

The samples of lyophilized gammaglobulin of CIG Batch-2 were analyzed by modified FPLC (Fast Protein Liquid Chromatography) using a Waters Alliance Liquid Chromatograph equipped with a Waters 996 photodiode array detector (scan 200-600 nm, quantitate 280 nm). The separation was accomplished using a Superdex 200 HR column (Supelco, Bellefonte, PA, part number 17-1088-01) with 50 mM sodium phosphate buffer (pH 7) mobile phase. Known standards of antibody fragments IgG, Fab, F(ab')₂, and Fc (Calbiochem, La Jolla, CA) were chromatographed for comparison of retention times. The F(ab')₂ was received in a solution with the concentration listed as 2.14 mg/mL protein. The IgG, Fab and Fc were received as dry powders. The IgG and Fab were diluted to a nominal concentration of 2 mg/mL with mobile phase. The Fc was diluted to a nominal concentration of 2.5 mg/mL with mobile phase. A detailed description of the analytical procedure is contained in Chemistry Method No. 14, February 13, 1998.

Standards

1. Human Immunoglobulin G, Plasma, IgG, Calbiochem, catalog number 401114, lot number B27643, sample number 394-34-15.
2. Human Immunoglobulin G, Fab Fragment, Plasma, Calbiochem, catalog number 401116, lot number B14394, sample number 394-35-04.
3. Human Immunoglobulin G, F(ab')₂ Fragment, Plasma, Calbiochem, catalog number 401103, lot number B27607, sample number 394-35-19.
4. Human Immunoglobulin G, Fc Fragment, Plasma, Calbiochem, catalog number 401104, lot number B29864, sample number 394-36-04.

Samples

The following sample was received for analysis. Approximately 20 mg of sample was weighed into a 10.0-mL volumetric flask and diluted to a nominal concentration of 2.0 mg/mL in mobile phase.

1. Lyophilized gammaglobulin – CIG Batch-2, Bottle #1, Lot D1138, expires 3/03.

Results

Tables 1-5 contain a list of the retention times and the relative percent area of peaks in the chromatograms of the IgG, Fab, F(ab')₂, Fc and sample. Chromatograms 1-5 are included after each corresponding table. The sample and standards were run in duplicate to verify reproducibility, however, the chromatograms and tables contain data from only the first injection. The %RSD between injections was less than 2.0% for the peak areas of the sample and standards.

Discussion

The sample contained peaks at the same retention time of the IgG monomer. The average area percent of this peak for the sample was 100.0 percent.

Table 1. IgG Std. 394-34-15 2 mg/mL Lot# B27643

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Component	-	0.09	15.52
IgG,Polymer	-	-	16.75
IgG,Dimer	4.70	4.66	20.00
IgG,Monomer	95.30	94.37	23.58
Low Molecular Weight Component	-	0.88	46.28

Figure 1. IgG Std. 394-34-15 2 mg/mL Lot# B27643

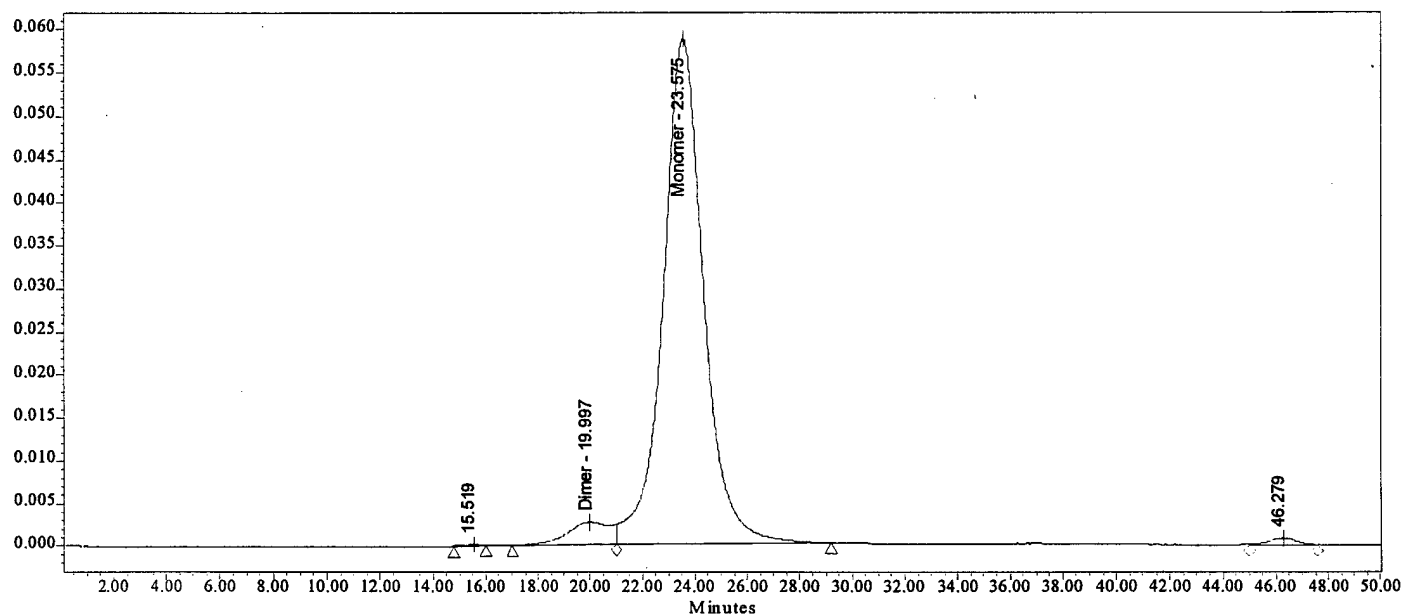


Table 2. Sample 1 – 394-36-20, CIG Batch-2, Bottle #1, Lot D1138

Tentative Identification	Relative Area Percent (polymer,dimer, monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Component	-	0.12	15.45
IgG,Polymer	-	-	16.75
IgG,Dimer	-	-	20.59
IgG,Monomer	100.00	99.47	23.55
Low Molecular Weight Component	-	0.42	39.65

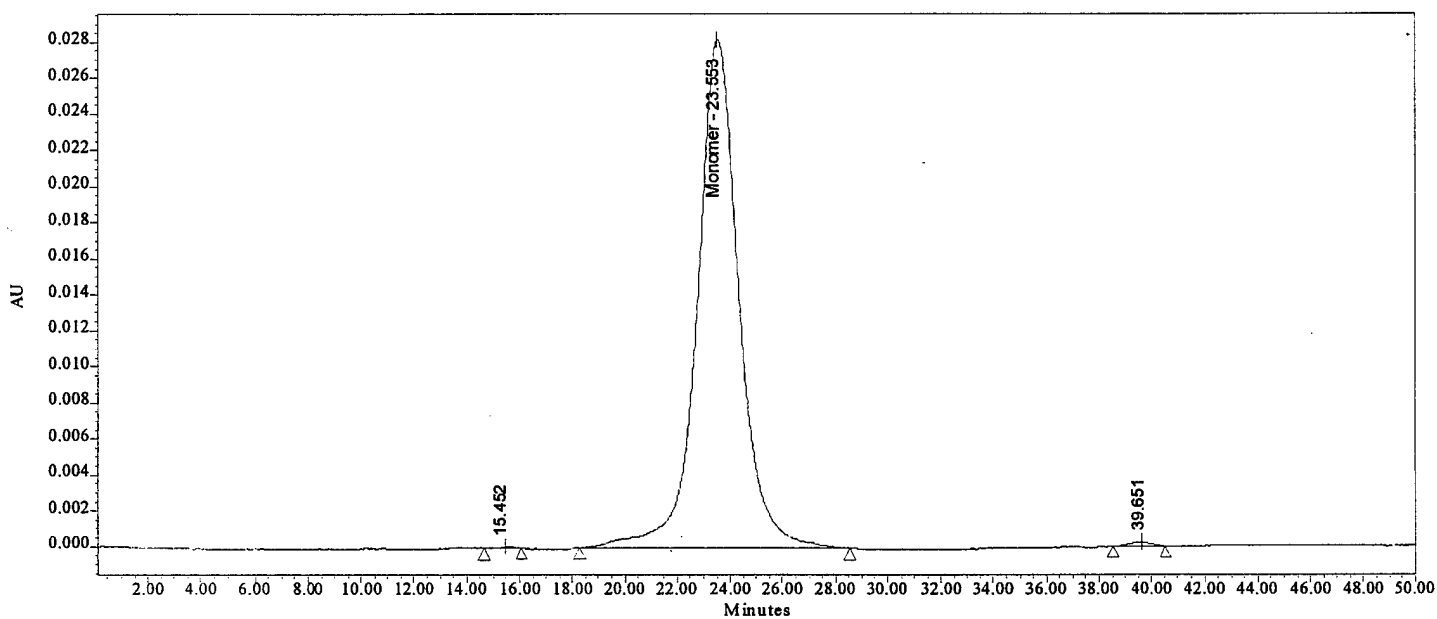
Figure 2. Sample 1 – 394-36-20, CIG Batch-2, Bottle #1, Lot D1138

Table 3. IgG, Fab Std. 394-35-04 2 mg/mL Lot# B14394

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
Fab	-	100.00	29.90

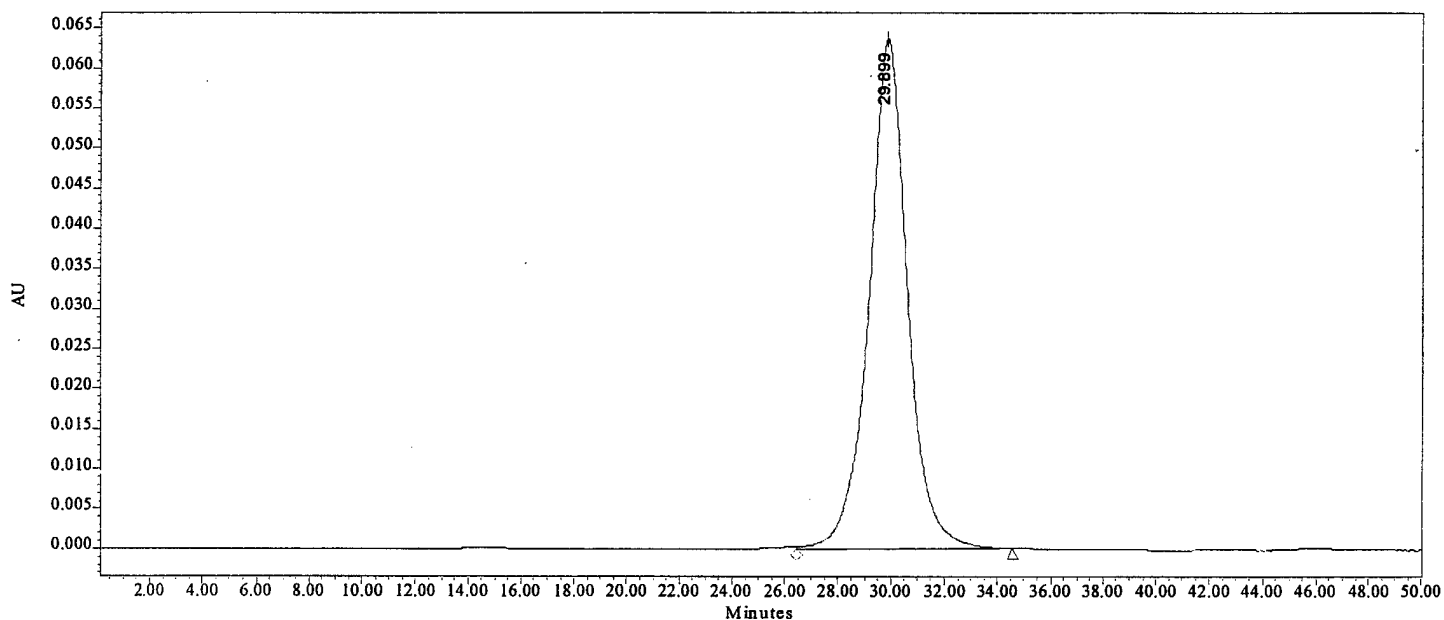
Figure 3. IgG, Fab Std. 394-35-04 2 mg/mL Lot# B14394

Table 6. IgG, F(ab')₂ Std. 394-35-19 2.14 mg/mL Lot# B27607

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Component	-	1.55	15.19
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
F(ab') ₂	-	97.43	25.53
Low Molecular Weight Component	-	1.02	32.03

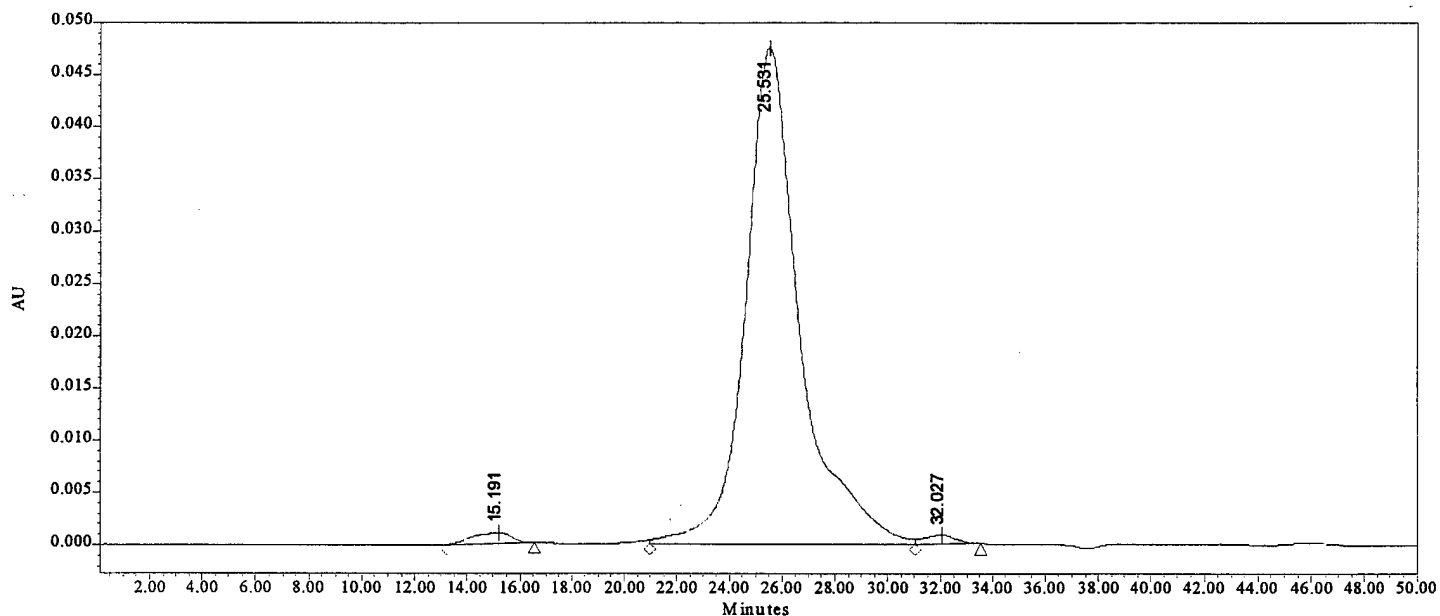
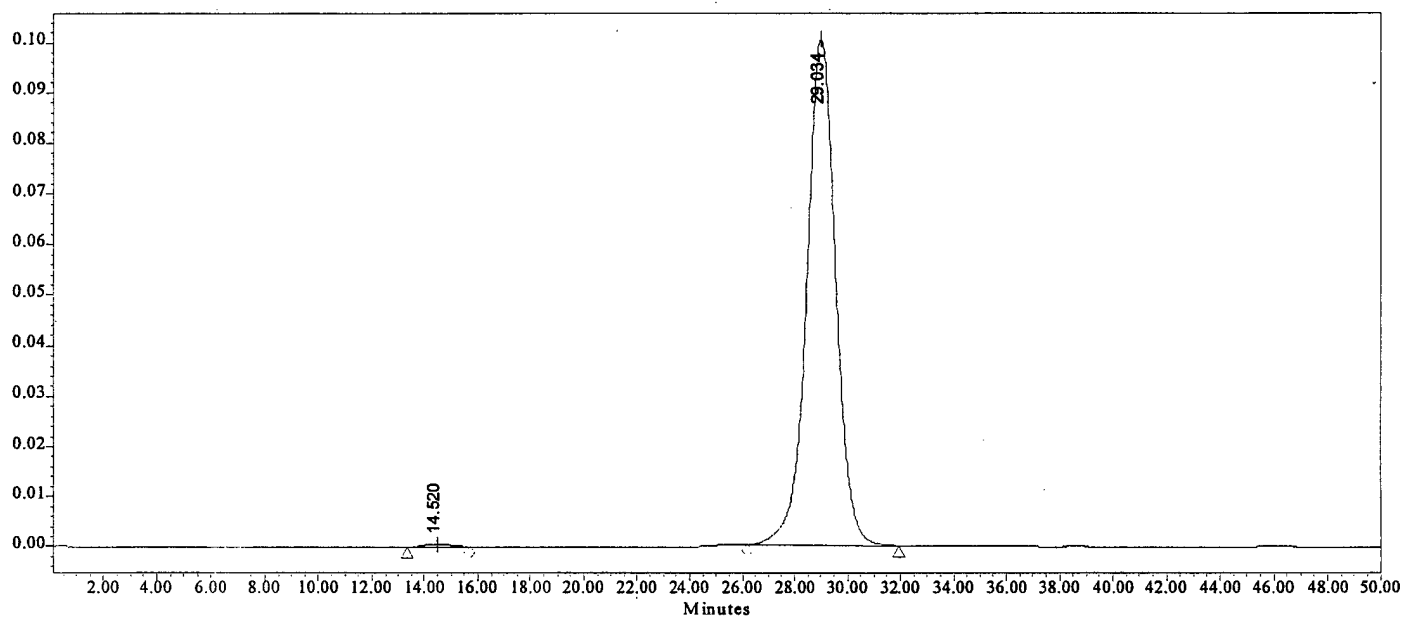
Figure 6. IgG, F(ab')₂ Std. 394-35-19 2.14 mg/mL Lot# B27607

Table 7. IgG, Fc Std. 394-36-04 2.5 mg/mL Lot# B29864

Tentative Identification	Relative Area Percent (polymer,dimer monomer only)	Relative Area Percent (total)	Retention Time (minutes)
High Molecular Weight Component	-	0.53	14.52
IgG,Polymer	-	-	-
IgG,Dimer	-	-	-
IgG,Monomer	-	-	-
Fc	-	99.47	29.03

Figure 7. IgG, Fc Std. 394-36-04 2.5 mg/mL Lot# B29864



APPENDIX G

Quantitative Analyses of Purified Immunoglobulins

LabCorp IG Analysis

Lot #	D1060		D1138		D1103		D1105		D1116		IVBG-1B		D1103/D1105	
Vial #	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Total Protein (g/dL)	3.5	3.5	3.7	4	3.9	3.9	3.7	3.9	3.4	3.7	12.2	12.2	3.8	3.9
Total IgG (mg/dL)	2703	2934	3267	3276	3315	3372	3233	3306	2981	3078	11722	11526	3199	3345
IgG-1 (mg/dL)	1536	1553	2073	2016	1890	1686	2082	2154	1814	1756	6309	6720	1980	2016
IgG-2 (mg/dL)	1314	1360	1908	1764	1800	2055	1362	1560	1179	1371	6558	5874	1551	1688
IgG-3 (mg/dL)	127	181	153	150	180	193	199	202	103	109	164	33	188	176
IgG-4 (mg/dL)	89	112	52	53	61	68	44	20	55	65	39	45	50	62
IgG (mg/dL)	2868	2805	3251	3222	3348	3478	3357	3380	3031	3161	9500	9430	3307	3443
IgA (mg/dL)	80	88	46	15	45	57	46	14	45	14	<4	<4	16	15
IgM (mg/dL)	16	15	<1	<1	4	4	2	2	<1	1	<1	<1	3	3

Lot D1060 = CIG*; Lot D1138 = CIG; Lot D1103 = PBIG; Lot D1105 = PBIG; Lot D1116 = BBIG; Lot IVBG-1B = BIG;

Lot D1103/D1105 = Combined PBIGAB; 2-vials were assayed from each lyophilized lot

* Lot D1060 was the only CIG used in the study.

Lot D1060 = Vial #1 was sent for analysis on 2/29/00. Vial #2 was sent for analysis on 6/7/00. The results from Vial #2 sent for analysis on 2/29/00 are not being reported due to an apparent error in the sample. The results from Vial #2 sent for analysis on 6/7/00 are not being reported due to an incomplete analysis of the sample by the testing facility.

REPORT GENERATED BY: MC 1-2-01

QC REVIEW MC 1-2-01

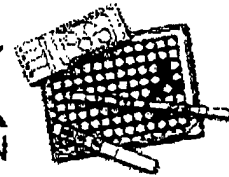
TECHNICAL REVIEW MC 1-2-01

VERIFIED EXACT
COPY
MC 1-2-01

APPENDIX H
Scimedx Corp. Reports

400 Ford Road -- Bldg. 100-- Denville, NJ 07834
973-625-8822 (phone) -- 973-625-8796 (fax)
scimedxinc@aol.com (e-mail)
www.scimedx.com (internet)

SCIMEDX
CORPORATION



Fax

To: Dr. Bob Hunt

From: Caryn M. Shapiro

Fax: 614 424-5469

Pages: 5

Attn:

Date: 2/3/00

Re: Lot to Lot Differences in Product

CC: Dr. Peter Guidon, Dr. Gary Lehnus

☐ **Urgent**

☐ **For Review**

☐ **Please Comment**

☐ **Please Reply**

☐ **Please Recycle**

Dear Bob,

I ran some spectrophotometric determinations on each of the individual lots of material that we have purified for you, and have included this data in a spreadsheet and several graphs. Gary Lehnus feels that while there will be a certain amount of procedural lot to lot variation, the large variations that we have now verified may very well be due to the starting material (plasma). He suggested that samples of all of the lots of starting material (plasma) could be analyzed for IgG values, to see if your immunization protocol enhanced the amount of IgG.

Please feel free to contact us if you need any further information.

Regards,

Caryn M. Shapiro

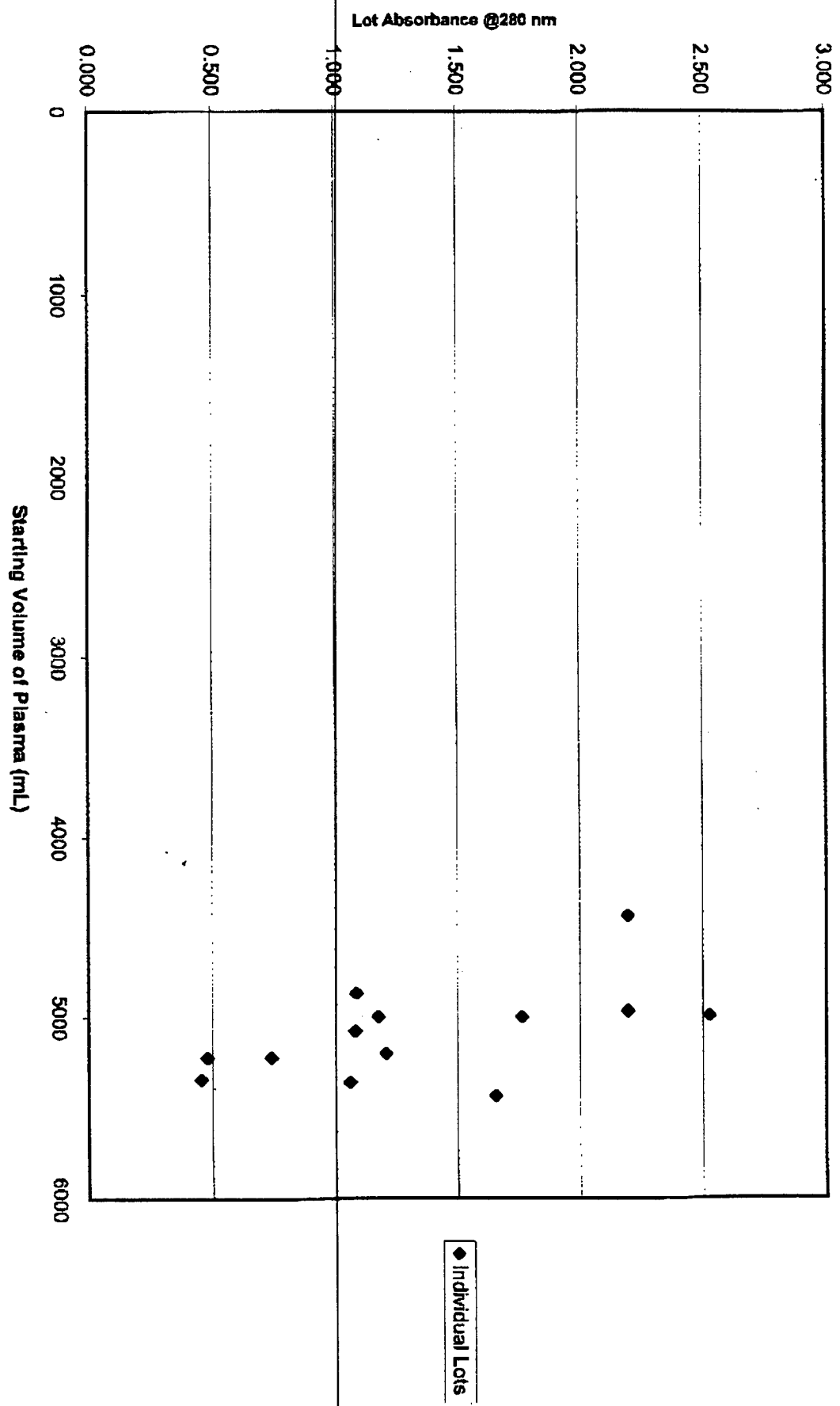


Battelle Lot Differences.xls

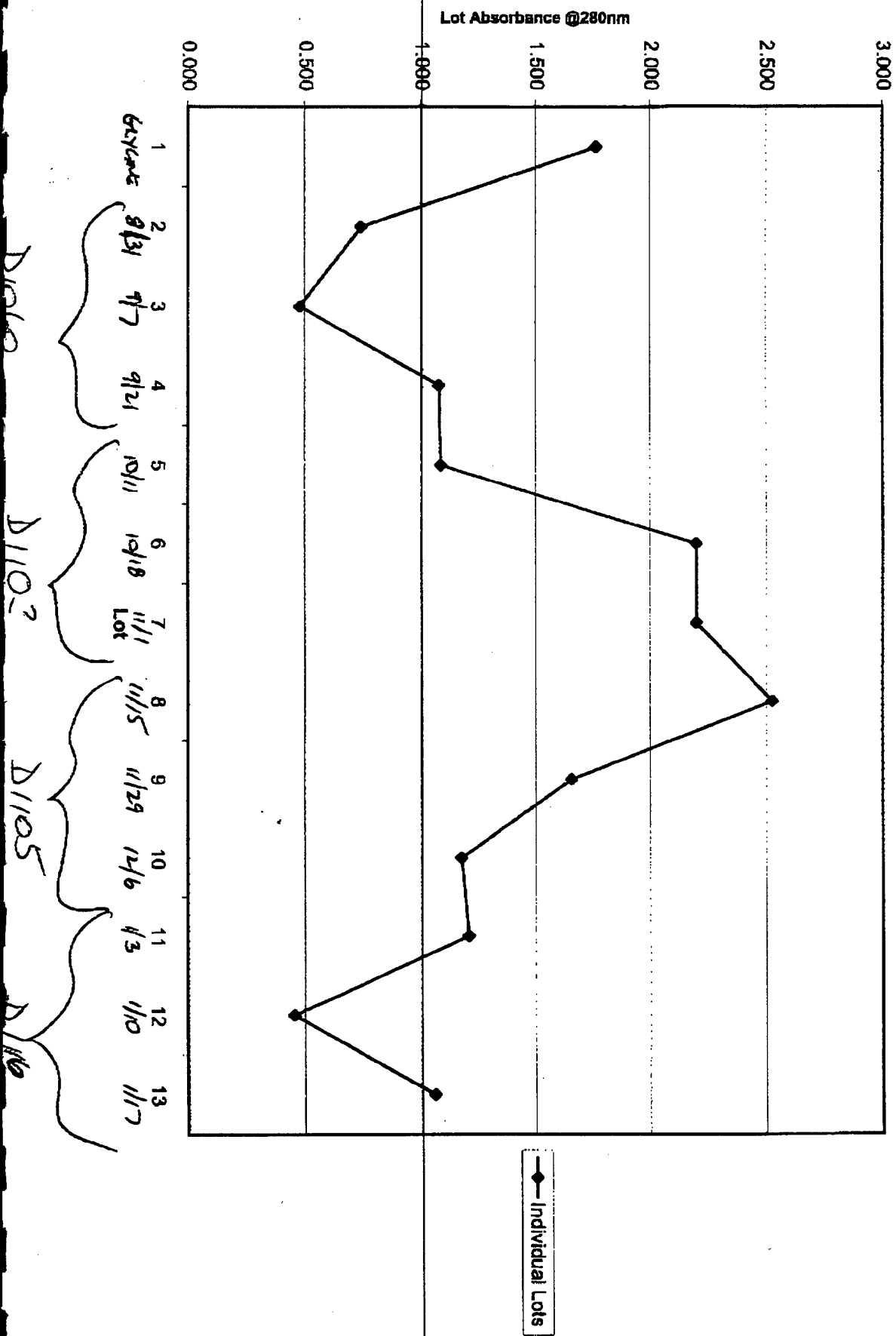
Lot	Starting Volume (mL)	Final Product (Abs@280)	Protein Concentration (mg/mL)	Final Product Lot	Final Product Protein Conc. (mg/mL)
Glycine	5000	1.764	35.8		
8/31/99	5230	0.739	15.1	D1060	~10.0
9/7/99	5230	0.480	9.8	D1060	~10.0
9/21/99	5080	1.078	21.9	D1060	~10.0
10/11/99	4870	1.084	22.0	D1103	37.0
10/18/99	4440	2.197	44.6	D1103	37.0
11/1/99	4970	2.197	44.6	D1103	37.0
11/15/99	4995	2.526	51.3	D1105	39.2
11/29/99	5440	1.655	33.5	D1105	39.2
12/6/99	5000	1.174	23.9	D1105	39.2
1/3/00	5205	1.205	24.4	D1116	13.3
1/10/00	5350	0.455	9.2	D1116	13.3
1/17/00	5365	1.058	21.4	D1116	13.3

PEACONCE LOT

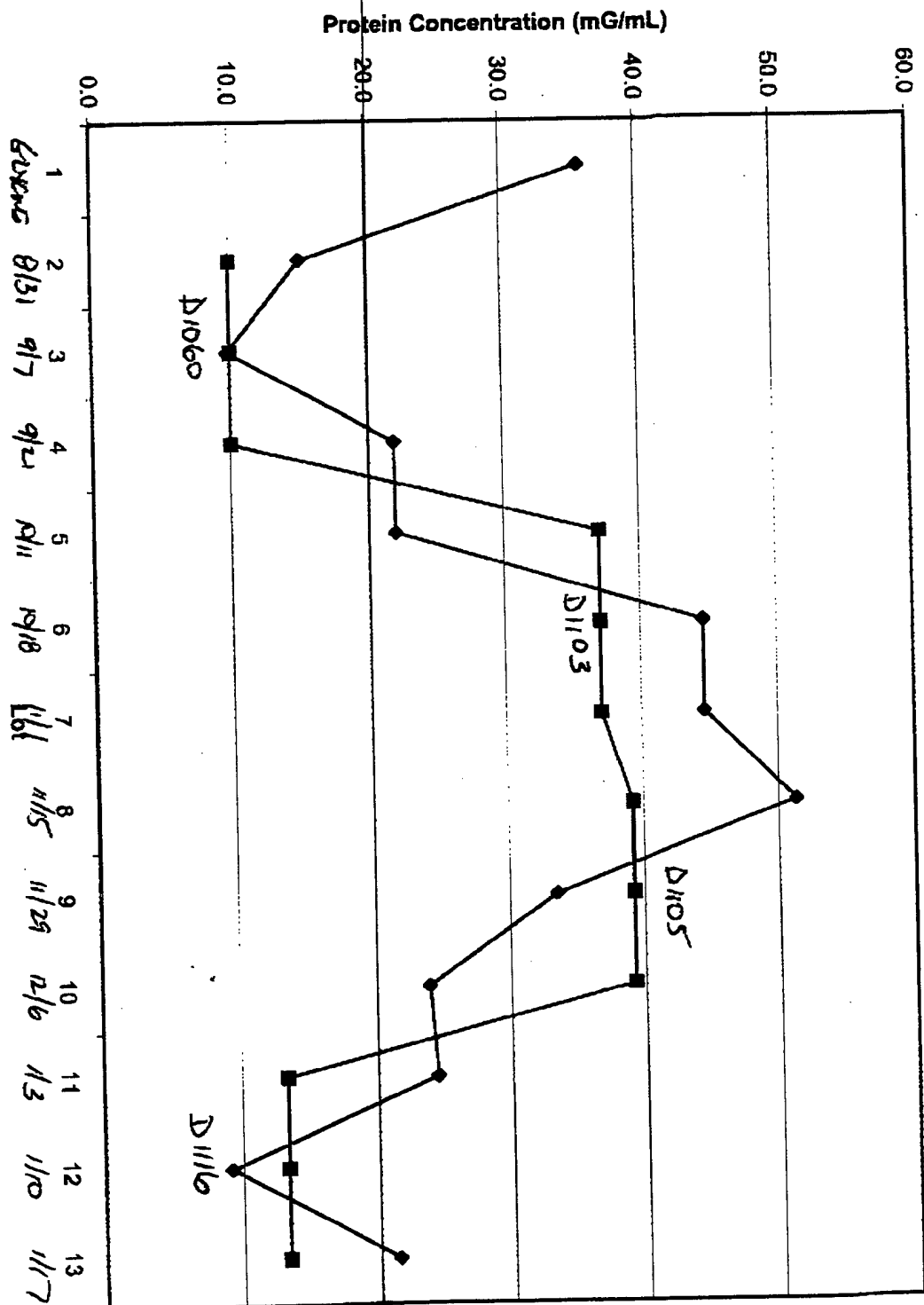
Battelle Lot Variations



Battelle Lot to Lot Absorbance Differences



Battelle Lot Differences



—◆— Individual Lots
—■— Final Product Lot

BIO*WHITTAKER

A CAMBREX Company

September 30, 1999

SCIMEDX Corp
Peter Guidon
400 Ford Road
Bldg. 100
Denville, NJ 07834

Dear Mr. Guidon,

Kinetic-QCL testing of your sample has been completed. The assay met all validation requirements per the FDA established guidelines for LAL testing. The results follow.

Sample Name	Lot #	Endotoxin Result
gammaglobulin solution	1	0.10 EU/ml
gammaglobulin solution	2	<0.05 EU/ml
gammaglobulin solution	3	<0.05 EU/ml
gammaglobulin solution	4	0.05 EU/ml

If you should have any questions concerning the results, please call us at 1-800-654-4452, extension 2251.

Best Regards,

Kathy Boyer

Kathy Boyer
Technical Analyst
Technical Services/LAL Testing Service
(LTS1221; 80-503, 80-509)

CIG Lot # D106D
MC
1-4-01

BIO*WHITTAKER

A CAMBREX Company

November 11, 1999

SCIMEDX Corp
Peter Guidon
400 Ford Road
Bldg. 100
Denville, NJ 07834

PB1GA Lot# D1103)
mc
1-4-01

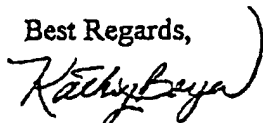
Dear Mr. Guidon,

Kinetic-QCL testing of your sample has been completed. The assay met all validation requirements per the FDA established guidelines for LAL testing. The results follow.

Sample Name	Lot #	Endotoxin Result
gammaglobulin	10/11/99	0.06 EU/ml
gammaglobulin	10/18/99	<0.05 EU/ml
gammaglobulin	11/01/99	0.05 EU/ml

If you should have any questions concerning the results, please call us at 1-800-654-4452, extension 2251.

Best Regards,



Kathy Boyer
Technical Analyst
Technical Services/LAL Testing Service
(LTS1270; 80-503, 80-509)

BIO*WHITTAKER

A CAMBREX Company

December 15, 1999

SCIMEDX Corp
Peter Guidon
400 Ford Road
Bldg. 100
Denville, NJ 07834

PB1GB (LOT#-D1105)
MC 1-4-01

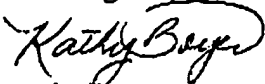
Dear Mr. Guidon,

Kinetic-QCL testing of your sample has been completed. The assay met all validation requirements per the FDA established guidelines for LAL testing. The results follow.

Sample Name	Lot #	Endotoxin Result
gammaglobulin	11/15/99	<0.05 EU/ml
gammaglobulin	11/29/99	<0.05 EU/ml
gammaglobulin	12/06/99	<0.05 EU/ml

If you should have any questions concerning the results, please call us at 1-800-654-4452, extension 2251.

Best Regards,



Kathy Boyer
Technical Analyst
Technical Services/LAL Testing Service
(LTS1301; 80-503, 80-509)

BIO*WHITTAKER**A CAMBRÉX Company**

January 28, 2000

Peter Guidon
SCIMEDX Corp
400 Ford Road
Bldg. 100
Denville, NJ 07834

BBIG (Lot# D11116)
MC 14-01

Dear Mr. Guidon,

Kinetic-QCL testing of your samples has been completed. The assay met all validation requirements per the FDA established guidelines for LAL testing. The results follow.

Sample Name	Lot #	Endotoxin Result
gammaglobulin	1/3/00	<0.05 EU/ml
gammaglobulin	1/10/00	<0.05 EU/ml
gammaglobulin	1/17/00	0.06 EU/ml

If you should have any questions concerning the results, please call us at 1-800-654-4452, extension 2251.

Best Regards,

Carol Roemer
Carol Roemer
LAL Testing Service Supervisor
(LTS1342; 80-503, 80-509)

BIO*WHITTAKER

A CAMBREX Company

March 22, 2000

Peter Guidon
SCIMEDX Corp
400 Ford Road
Bldg. 100
Denville, NJ 07834

Dear Mr. Guidon,

Kinetic-QCL testing of your samples has been completed. The assay met all validation requirements per the FDA established guidelines for LAL testing. The results follow.

Sample Name	Lot #	Endotoxin Result
gammaglobulin	2/14/00	<0.05 EU/ml
gammaglobulin	2/28/00	<0.05 EU/ml
gammaglobulin	3/13/00	<0.05 EU/ml ✓

If you should have any questions concerning the results, please call us at 1-800-654-4452, extension 2251.

Best Regards,

Kathy Boyer

Kathy Boyer
Technical Analyst
LAL Testing Service/Technical Services
(LTS1418; 80-503, 80-509)

WU 1-4-01 MC
C/C Lot # D1138
MC 1-4-01

APPENDIX I

List of Plasma Samples Sent to Scimedx Corp. for Purification and Lyophilization

TASK G155553A PLASMA SAMPLES SENT TO SCIMEDX CORPORATION FOR PROCESSING

<u>Subject ID</u>	<u>Date Received</u>	<u>Timepoint</u>	<u>BarCode</u>	<u>Bleed</u>	<u>Donor</u>	<u>Date Sent</u>
004	08/11/1998	0	98155503789	0330713489	0330340905	07/06/1999
018	08/11/1998	0	98155503793	0330713493	0330340948	07/06/1999
023	08/11/1998	0	98155503791	0330713492	0330340930	07/06/1999
034	08/11/1998	0	98155503788	0330713488	0330340891	07/06/1999
036	08/11/1998	0	98155503790	0330713491	0330340921	01/31/2000
038	08/11/1998	0	98155503792	0330713490	0330340913	07/06/1999
127	08/11/1998	0	98154499178	0330714266	0330341120	07/06/1999
154	08/11/1998	0	98154499179	0330714227	0330341138	07/06/1999
250	08/14/1998	0	98159506255	0480662664	04801493	01/31/2000
263	08/14/1998	0	98159506254	0480662660	04801971	01/31/2000
249	08/14/1998	0	98159506256	0480662667	0480102	01/31/2000
295	08/25/1998	0	98159506260	0480666354	04801824	01/31/2000
285	08/25/1998	0	98159506259	0480666346	04801653	01/31/2000
283	08/25/1998	0	98159506258	0480666343	0480357	01/31/2000
267	08/25/1998	0	98159506261	0480666371	04802099	01/31/2000
272	08/25/1998	0	98159506262	0480666703	0480523	01/31/2000
170	08/27/1998	0	98154499182	0330715684	0330341464	07/06/1999
168	08/27/1998	0	98154499183	0330715685	0330341472	07/06/1999
167	08/27/1998	0	98154499181	0330715683	0330341456	07/06/1999
052	08/27/1998	0	98155503797	0330714953	0330341375	07/06/1999
045	08/27/1998	0	98155503795	0330714951	0330341359	07/06/1999
060	08/27/1998	0	98155503794	0330714950	0330341341	07/06/1999
066	08/27/1998	0	98155503796	0330714952	0330341367	07/06/1999
099	09/24/1998	0	98155503801	0330716409	0330341642	07/06/1999
074	09/24/1998	0	98155503798	0330716406	0330341618	07/06/1999
179	09/24/1998	0	98154499184	0330718699	0330342711	07/06/1999
085	09/24/1998	0	98155503799	0330716408	0330341634	07/06/1999
083	09/24/1998	0	98155503800	0330716407	0330341626	07/06/1999
299	09/29/1998	0	98159506266	0480671162	04803152	01/31/2000
316	09/29/1998	0	98159506267	0480671172	04803053	01/31/2000
319	09/30/1998	0	98159506265	0480671158	04803159	01/31/2000
303	09/30/1998	0	98159506263	0480671148	04802690	01/31/2000
335	11/05/1998	0	98159506271	0480675633	0480335	01/31/2000
336	11/05/1998	0	98159506272	0480675627	04803588	01/31/2000
342	11/05/1998	0	98159506268	0480675823	0480342	01/31/2000
246	12/10/1998	112	98159506277	0480682255	0480430748	10/04/1999
258	12/10/1998	112	98159506273	0480681863	0480430462	10/26/1999
254	12/10/1998	112	98159506283	0480681667	0480430322	10/04/1999
252	12/10/1998	112	98159506284	0480681860	0480430357	10/26/1999
251	12/10/1998	112	98159506281	0480682442	0480430861	10/26/1999
266	12/10/1998	112	98159506275	0480681857	0480430454	10/26/1999
036	12/22/1998	0	98155503826	0330727363	0330348035	01/31/2000
189	12/22/1998	0	98155503805	0330721975	0330344498	01/31/2000
200	12/22/1998	0	98155503806	0330721990	0330344528	01/31/2000
107	12/22/1998	0	98155503803	0330721927	0330344455	01/31/2000
118	12/22/1998	0	98155503804	0330721945	0330304763	07/06/1999
121	12/22/1998	112	98154499218	0330728277	0330348485	10/26/1999
050	12/22/1998	112	98155503827	0330728334	0330348566	10/26/1999
056	12/22/1998	112	98155503833	0330728793	0330348868	10/04/1999
072	12/22/1998	112	98155503829	0330728659	0330348779	10/26/1999
069	12/22/1998	112	98155503832	0330728751	0330348841	10/04/1999
015	12/22/1998	112	98155503824	0330727058	0330347756	10/04/1999
026	12/22/1998	112	98155503819	0330726990	0330347675	10/26/1999
153	12/22/1998	112	98154499216	0330728127	0330348361	10/04/1999
135	12/22/1998	112	98154499215	0330728120	0330348345	10/04/1999
008	12/22/1998	112	98155503818	0330726703	0330347489	10/04/1999
289	01/19/1999	112	98159506293	0480685428	0480432457	10/26/1999
287	01/19/1999	112	98159506289	0480685274	0480432341	10/26/1999
286	01/19/1999	112	98159506301	0480684723	0480431990	10/04/1999
284	01/19/1999	112	98159506287	0480685261	0480432325	10/04/1999
321	01/19/1999	112	98159506315	0480688828	0480434263	10/04/1999
343	02/16/1999	112	98159506319	0480693053	0480436819	10/04/1999
339	02/16/1999	112	98159506320	0480693062	0480436827	10/26/1999

Report Generated: MC 1-2-01

QC REVIEW MC 1-2-01

TECHNICAL REVIEW MC 1-2-01

VERIFIED

COPY
MC 1-2-01

TASK G155553A PLASMA SAMPLES SENT TO SCIMEDX CORPORATION FOR PROCESSING

<u>Subject ID</u>	<u>Date Received</u>	<u>Timepoint</u>	<u>BarCode</u>	<u>Bleed</u>	<u>Donor</u>	<u>Date Sent</u>
308	02/16/1999	112	98159506321	0480693069	0480436835	10/04/1999
344	04/06/1999	0	98159506334	0480696365	0480438790	01/31/2000
354	04/06/1999	0	98159506335	0480696368	0480438803	01/31/2000
338	04/06/1999	112	98159506330	0480693469	0480437033	10/04/1999
352	04/06/1999	0	98159506336	0480696381	0480438811	01/31/2000
120	05/04/1999	112	98155503866	0330735487	0330352229	10/04/1999
092	05/04/1999	112	98155503845	0330730514	0330349911	10/04/1999
217	05/04/1999	112	98155503884	0330736101	0330352512	10/26/1999
175	05/04/1999	112	98155503888	0330732467	0330350960	10/26/1999
088	05/04/1999	112	98155503844	0330730474	0330349881	10/04/1999
171	05/04/1999	112	98155503890	0330732483	0330350978	10/26/1999
184	05/04/1999	0	98155503854	0330733712	0330351524	01/31/2000
100	05/04/1999	112	98155503868	0330735517	0330352237	10/04/1999
091	05/04/1999	112	98155503841	0330730113	0330349597	10/26/1999
138	05/04/1999	112	98155503835	0330729965	0330349511	10/26/1999
103	05/04/1999	112	98155503865	0330735677	033032296	10/26/1999
033	05/04/1999	112	98155503811	0330726571	0330347349	10/04/1999
108	05/04/1999	112	98155503869	0330735481	0330352211	10/26/1999
078	05/04/1999	112	98155503839	0330730083	0330303775	10/04/1999
222	05/04/1999	112	98155503886	0330736110	0330352521	10/04/1999
075	05/04/1999	112	98155503843	0330730436	0330349872	10/26/1999
080	05/04/1999	112	98155503837	0330729989	0330349546	10/26/1999
156	05/04/1999	112	98155503838	0330729995	0330349554	10/26/1999
032	05/04/1999	112	98155503812	0330726588	0330347381	10/26/1999
005	12/14/1999	210	98155503861	0330738684	0330249096	12/15/1999
006	12/14/1999	210	98155503863	0330738205	0330353454	12/15/1999
017	12/14/1999	210	98155503870	0330738350	0330353497	12/15/1999
020	12/14/1999	210	99165115128	0330753113	0330358391	12/15/1999
028	12/14/1999	210	98155503871	0330738362	0330322958	12/15/1999
053	12/14/1999	210	98155503895	0330739982	0330332741	12/15/1999
061	12/14/1999	210	98155503877	0330739947	0330354272	12/15/1999
063	12/14/1999	210	98155503876	0330740178	0330354388	12/15/1999
094	12/14/1999	210	98155503896	0330741950	0330340891	12/15/1999
104	12/14/1999	210	98165115124	0330760372	0330357000	12/15/1999
109	12/14/1999	210	98165115123	0330760105	0330357085	12/15/1999
128	12/14/1999	210	98155503880	0330741635	0330355015	12/15/1999
142	12/14/1999	210	98155503897	0330739838	0330354230	12/15/1999
155	12/14/1999	210	98155503898	0330739822	0330354205	12/15/1999
178	12/14/1999	210	98155503900	0330745253	0330356160	12/15/1999
228	12/14/1999	210	99165115134	0330358561	0330753509	12/15/1999
265	12/14/1999	210	98159506337	0480706431	0480444242	12/15/1999
330	12/14/1999	210	98159506338	0480706438	0480444251	12/15/1999
345	12/14/1999	210	98159506339	0480706429	0480444234	12/15/1999
027	12/14/1999	210	98155503892	0330748782	0330336681	12/15/1999
364	12/14/1999	0	98155503902	0330739011	0330353829	01/31/2000

REPORT GENERATED BY: MC 1-2-01

QC REVIEW MC 1-2-01

TECHNICAL REVIEW MC 1-2-01

VERIFIED EXACT
COPY
mc 1-2-01

APPENDIX J

Immune Globulin NAC Determination (U/ML)

Immune Globulin NAC Determination (U/mL)

007
1/3/01

Serotype	PBIGA	PBIGB	PBIGAB	BBIG	CIG
A	2.9	1.7	2.0	1.3	*
B	0.12	0.30	0.25	0.10	*
C	0.78	0.96	0.85	0.51	*
D	1.8	0.68	1.8	0.46	*
E	0.051	0.030	0.067	0.060	*

* denotes titer below the Limit Of Quantitation (LOQ)

APPENDIX K

Validation of Antitoxin Standards for Serotypes A, C, and D



Project Number G1555-53ASTAT (3104)

Date June 16, 1999

To Bob Hunt (JM-3)

From Nancy Niemuth

Subject **Validation of Antitoxin Standards for Serotypes A, C, and D**

Internal Distribution

Dept. Files
BA Starner (JM-3)
NA Niemuth
MC Matthews
JR Holdcraft
RMO

s:\niem\B13\Task53\VOS memo.doc +
Validation of standards.doc

The attached report summarizes validation experiments for the new (1998 lyophilized) standards for botulinum serotypes A, C, and D. The statistical analysis is limited to head-to-head comparison, serospecificity, and avidity experiments, although additional material provided by Tom Gelzleichter is included in Appendix B and was utilized in making recommendations. A draft version of this report was reviewed by Tom Gelzleichter before he left Battelle and he concurred on the conclusions and recommendations at that time.

NAN:llj
Attachment

For Review and Approval

	Name	Internal	Date
Originator	Nancy Niemuth	N	6/16/99
Concurrence	Jennifer Holdcraft	JRH	6/16/99
Approved	Bill Rosebrough	WRE	6/16/99

Sent Via: Interoffice Mail

STATISTICAL REPORT ON VALIDATION OF STANDARDS

INTRODUCTION

Botulinum antitoxin standards are required for mouse neutralization experiments conducted under MREF Task 97-53 and other botulinum toxin research. Insufficient quantities of the 1996 liquid standard preparations for serotypes A, C, and D were available to complete the planned Task 97-53 experiments. Thus, new standards were required for use in Task 97-53 experiments and other botulinum studies. PerImmune, Inc., 1998 standards are a new issue of the 1996 product in lyophilized form. A validation study was conducted to evaluate the new standard preparation for each serotype in comparison to the old standard. Serospecificity, avidity, and head-to-head comparison experiments are summarized in this report. Statistical comparisons between the old and new standard preparations were made for the head-to-head comparison experiments.

As noted below, these experiments showed that the liquid and lyophilized standards for serotypes A and C were comparable, but the new lyophilized standard for serotype D appeared to be substantially different from the old liquid standard. Additional experiments were conducted at Battelle and PerImmune to further characterize the serotype D standards. These additional tests are summarized in Appendix B.

METHODS

Probit models were fitted to the antitoxin dose-lethality response data for head-to-head comparison experiments. Model parameters were used to estimate the ED_{50} for each experiment. Paired t-tests were used to compare mean ED_{50} s for old and new standard preparations for each serotype.

A Likelihood Ratio Test (LRT) was used to determine whether the dose-response curves could adequately be described using a common slope and intercept within each serotype and standard preparation. A common slope and intercept would indicate that day-to-day assay variability was negligible.

RESULTS

Table 1 presents the probit slope, intercept, and estimated ED_{50} for each experiment. The raw data for these experiments are listed in Appendix A, Table A-1. LRTs indicated that day-to-day variability was substantial for serotypes A and D, with p-values of 0.002 and 0.007, respectively, for the old standard; and p-values of <0.001 and 0.006 for the new standard. Thus, a common slope was estimated for each combination of serotype and antitoxin standard, with separate intercepts for each experiment. Figures 1 through 3 present the probit models for each serotype.

Table 2 presents descriptive statistics for old (1996 liquid) and new (1998 lyophilized) standards, along with paired t-test results. The mean ED_{50} s for serotypes A and C were similar

and the difference in means was not statistically significant. For serotype D, the mean for the 1998 lyophilized standard was nearly double that of the 1996 liquid standard, a statistically significant increase ($p=0.012$). To explore this apparent difference for serotype D, additional experiments were conducted to further characterize potency of the old and new standards and to compare potency with the original results reported in the Task 95-39 final report (chemical characterization of standards). These additional tests are summarized in Appendix B.

Results of serospecificity experiments for the 1998 antitoxin standard preparations are presented in Table 3. The raw data for these experiments are listed in Appendix A, Table A-2. Each antitoxin standard protected 100 percent of the mice exposed to the specified serotype and generally failed to protect the mice exposed to other serotypes. For the serotype A standard, 2/4 mice survived the exposure to 50 MIP LD₅₀ units of serotype C toxin. For the serotype A and C standards, 1 of the 4 mice survived exposure to serotype E toxin.

Results of avidity experiments are presented in Tables 4a and 4b. The raw data for these experiments are listed in Appendix A, Table A-3. Average potency values for Task 53 potency experiments were calculated and used in the toxin dose calculation (MIP LD₅₀ units/injection) for the avidity experiments. For serotypes A and D, the number of MIP LD₅₀ units per injection (Table 4a) for the new standard was similar to that of the old standard as reported in Task 39 and concurrent experiments in Task 53. For serotype C, the number of MIP LD₅₀ units per injection for the new standard appeared to be substantially reduced compared to the old. Avidity ratios, however, were consistent over the three sets of experiments for all serotypes (Table 4b).

CONCLUSIONS AND RECOMMENDATIONS

For serotypes A and C, the mean ED₅₀s for the old and new standard preparations were similar. For serotype D, the mean for the 1998 lyophilized standard was nearly double that of the 1996 liquid standard, a statistically significant increase ($p=0.012$). Each antitoxin standard protected 100 percent of the mice exposed to the specified serotype and generally failed to protect the mice exposed to other serotypes. Avidity ratios were consistent over experiments conducted in Tasks 39 and 53, although the number of MIP LD₅₀ units per injection for the lyophilized standard for serotype C appeared to be substantially reduced compared to the 1996 liquid standard.

We make the following recommendations:

1. As stability of the 1996 internal standard for serotype D is not unequivocally demonstrated, this standard should no longer be used in-house. The 1996 standards have not been in use for the neutralization assay since March 1998, for any serotype.
2. The 1998 lyophilized standards for serotypes A, C, and D should be adopted for use on Task 53, Task 161, and future studies.
3. The PerImmune calibration values for serotype D should be adopted.
4. No change in calibration values is necessary for serotypes A and C.

As described in Appendix B, recommendations for serotype D are based on (1) the internal consistency of PerImmune serotype D results with their other serotypes, (2) a trend towards increasing potency during stability testing of the 1996 liquid standard at Battelle, and (3) an apparent increase in the 1996 liquid standard concentration at Battelle, as measured by UV absorption. In addition, the PerImmune calibration values were tested to identify the amount of toxin that is 50 percent neutralized by the standard antitoxin amount (0.023 U/ml). These values were in agreement with the original 1996 values (i.e., 27 MIP LD₅₀).

Table 1. Summary of Head-to-Head Comparison Experiments

Serotype	Date	Experiment Number	Standard	Slope	Intercept	ED ₅₀
A	08/03/98	7013	Old	-6.16	-9.99	0.024
A	08/04/98	7022	Old	-6.16	-10.4	0.020
A	08/05/98	7031	Old	-6.16	-9.49	0.029
A	08/06/98	7040	Old	-6.16	-9.34	0.030
A	08/03/98	7014	New	-6.91	-13.0	0.013
A	08/04/98	7023	New	-6.91	-11.5	0.022
A	08/05/98	7032	New	-6.91	-12.1	0.018
A	08/06/98	7041	New	-6.91	-10.9	0.026
C	08/04/98	7024	Old	-4.80	-8.58	0.016
C	08/05/98	7033	Old	-4.80	-8.58	0.016
C	08/06/98	7042	Old	-4.80	-8.67	0.016
C	10/14/98	7179	Old	-4.80	-8.72	0.015
C	08/04/98	7025	New	-3.99	-6.86	0.019
C	08/05/98	7034	New	-3.99	-6.72	0.021
C	08/06/98	7043	New	-3.99	-7.47	0.013
C	10/14/98	7180	New	-3.99	-6.98	0.018
D	08/04/98	7026	Old	-5.82	-8.90	0.030
D	08/05/98	7035	Old	-5.82	-9.60	0.022
D	09/01/98	7128	Old	-5.82	-9.86	0.020
D	09/03/98	7131	Old	-5.82	-9.38	0.024
D	08/04/98	7027	New	-5.84	-7.92	0.044
D	08/05/98	7036	New	-5.84	-7.92	0.044
D	09/01/98	7129	New	-5.84	-8.31	0.038
D	09/03/98	7132	New	-5.84	-7.29	0.056

Table 2. Descriptive Statistics and Statistical Comparison of Head-to-Head Comparison Experiments

Serotype	1996 Liquid Standard (Old)		1998 Lyophilized Standard		P-value for Difference in Means
	Mean ED ₅₀	SD	Mean ED ₅₀	SD	
A	0.026	0.005	0.020	0.006	0.140
C	0.016	0.001	0.018	0.003	0.280
D	0.024	0.004	0.046	0.008	0.012

Table 3. Summary of Serospecificity Experiments for 1998 Standards

Botulinum Serotype	Antitoxin Standard A			Antitoxin Standard C			Antitoxin Standard D		
	Toxin Dose (MIP LD ₅₀)	# surv #tested	Percent Protected (%)	Toxin Dose (MIP LD ₅₀)	# surv #tested	Percent Protected (%)	Toxin Dose (MIP LD ₅₀)	# surv #tested	Percent Protected (%)
A	1000	4/4	100	100	0/4	0	50	0/4	0
B	50	0/4	0	100	0/4	0	50	0/4	0
C	50	2/4	50	2000	4/4	100	500	0/4	0
D	50	0/4	0	1000	0/4	0	1000	4/4	100
E	50	1/4	25	100	1/4	25	50	0/4	0

Table 4a. Number of MIP LD₅₀ Units per Injection for Three Sets of Experiments at Three Test Levels, for Serotypes A, C, and D

Serotype	Level of Test	Number of MIP LD ₅₀ Units per Injection at Test Level		
		Task 39 Old Standard	Task 53 Old Standard	Task 53 New Standard
A	L+/10	1400	1032	1414
	L+/33	230	211	244
	L+/100	23	24	29
C	L+/10	1600	1226	686
	L+/33	360	304	218
	L+/100	69	36	24
D	L+/10	2100	1852	2300
	L+/33	260	254	202
	L+/100	25	21	24

Table 4b. Avidity Ratios for Three Sets of Experiments at Three Test Levels, for Serotypes A, C, and D

Serotype	Test Ratio	Avidity Ratio		
		Task 39 Old Standard	Task 53 Old Standard	Task 53 New Standard
A	[L+/10] / [3.3*L+/33]	1.8	1.5	1.8
	[L+/33] / [3*L+/100]	3.2	2.9	2.8
	[L+/10] / [10*L+/100]	5.8	4.2	5.0
C	[L+/10] / [3.3*L+/33]	1.3	1.2	1.0
	[L+/33] / [3*L+/100]	1.7	2.8	3.1
	[L+/10] / [10*L+/100]	2.3	3.4	2.9
D	[L+/10] / [3.3*L+/33]	2.5	2.2	3.4
	[L+/33] / [3*L+/100]	3.5	4.1	2.8
	[L+/10] / [10*L+/100]	8.5	9.0	9.4

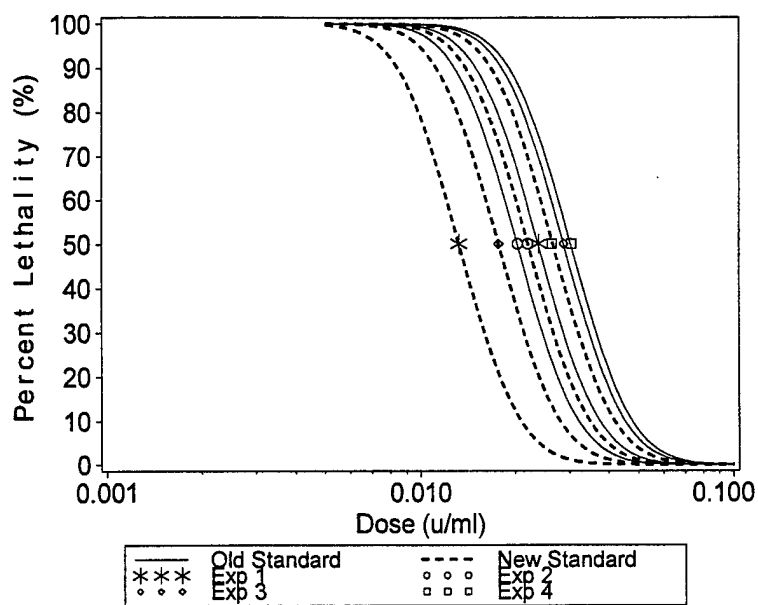


Figure 1. Modeled Dose-Response Curves for Head-to-Head Comparison Experiments for Serotype A

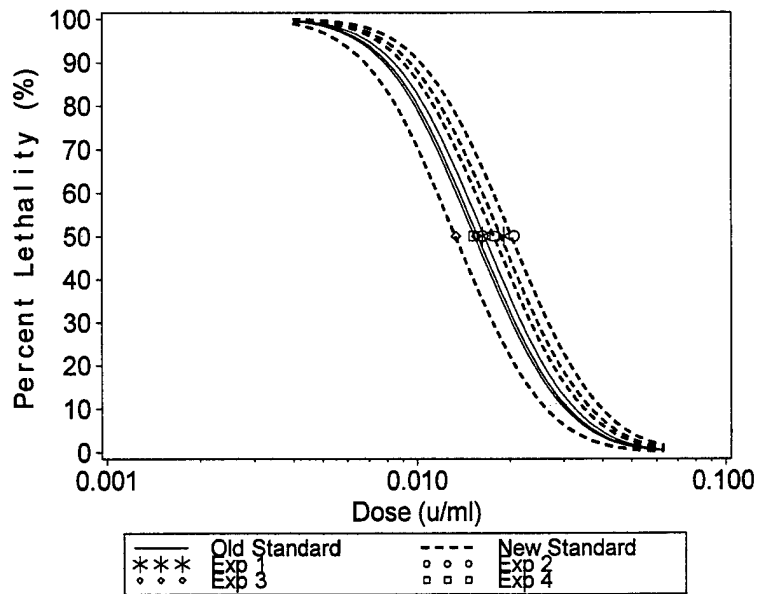


Figure 2. Modeled Dose-Response Curves for Head-to-Head Comparison Experiments for Serotype C

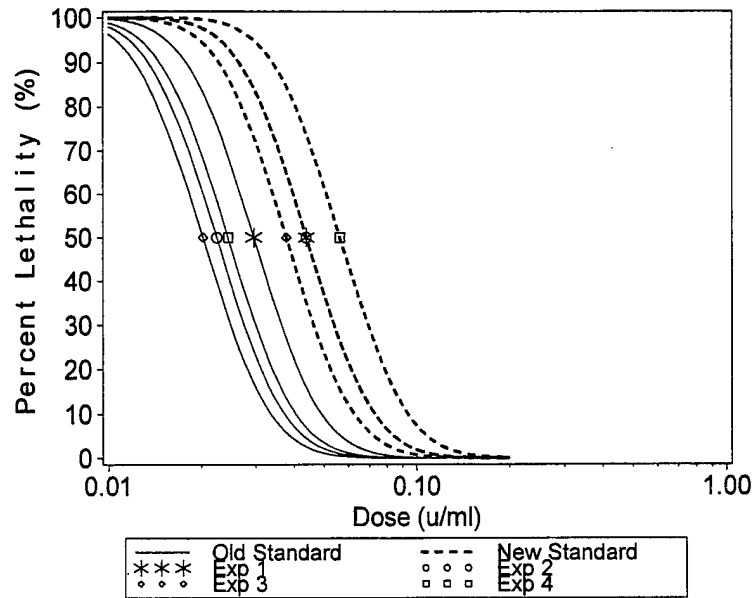


Figure 3. Modeled Dose-Response Curves for Head-to-Head Comparison Experiments for Serotype D

APPENDIX A:
RAW DATA LISTINGS

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
A	08/03/98	7013	Old	0.008	7 / 8	87.500
				0.012	7 / 7	100.000
				0.017	7 / 8	87.500
				0.024	7 / 8	87.500
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/03/98	7014	New	0.008	6 / 8	75.000
				0.012	4 / 8	50.000
				0.017	5 / 8	62.500
				0.024	0 / 8	0.000
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/04/98	7022	Old	0.008	7 / 8	87.500
				0.012	8 / 8	100.000
				0.017	8 / 8	100.000
				0.024	2 / 8	25.000
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/04/98	7023	New	0.008	8 / 8	100.000
				0.012	8 / 8	100.000
				0.017	8 / 8	100.000
				0.024	2 / 8	25.000
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/05/98	7031	Old	0.008	8 / 8	100.000
				0.012	8 / 8	100.000
				0.017	8 / 8	100.000
				0.024	7 / 8	87.500
				0.034	1 / 8	12.500
				0.048	0 / 8	0.000
A	08/05/98	7032	New	0.008	8 / 8	100.000
				0.012	8 / 8	100.000
				0.017	4 / 8	50.000
				0.024	1 / 8	12.500
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/06/98	7040	Old	0.008	8 / 8	100.000
				0.012	7 / 8	87.500
				0.017	8 / 8	100.000
				0.024	7 / 8	87.500
				0.034	4 / 8	50.000
				0.048	0 / 8	0.000
A	08/06/98	7041	New	0.008	8 / 8	100.000
				0.012	7 / 8	87.500
				0.017	7 / 8	87.500
				0.024	8 / 8	100.000
				0.034	1 / 8	12.500
				0.048	0 / 8	0.000

MREF Task 97-53

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
C	08/04/98	7024	Old	0.005	8 / 8	100.000
				0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	7 / 8	87.500
				0.018	2 / 8	25.000
				0.026	1 / 8	12.500
C	08/04/98	7025	New	0.005	8 / 8	100.000
				0.006	7 / 8	87.500
				0.009	7 / 8	87.500
				0.013	8 / 8	100.000
				0.018	5 / 8	62.500
				0.026	1 / 8	12.500
C	08/05/98	7033	Old	0.005	8 / 8	100.000
				0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	7 / 8	87.500
				0.018	2 / 8	25.000
				0.026	1 / 8	12.500
C	08/05/98	7034	New	0.005	8 / 8	100.000
				0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	7 / 8	87.500
				0.018	5 / 8	62.500
				0.026	2 / 8	25.000
C	08/06/98	7042	Old	0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	6 / 6	100.000
				0.018	2 / 8	25.000
				0.026	0 / 8	0.000
C	08/06/98	7043	New	0.005	8 / 8	100.000
				0.006	6 / 8	75.000
				0.009	5 / 7	71.429
				0.013	5 / 8	62.500
				0.018	2 / 8	25.000
				0.026	2 / 8	25.000
C	10/14/98	7179	Old	0.005	7 / 8	87.500
				0.006	7 / 8	87.500
				0.009	7 / 8	87.500
				0.013	5 / 8	62.500
				0.018	5 / 8	62.500
				0.026	2 / 8	25.000
				0.037	0 / 8	0.000
C	10/14/98	7180	New	0.005	8 / 8	100.000
				0.006	7 / 8	87.500
				0.009	7 / 8	87.500
				0.013	8 / 8	100.000
				0.018	5 / 8	62.500

MREF Task 97-53

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
C	10/14/98	7180	New	0.026	1 / 8	12.500
				0.037	0 / 8	0.000
				0.052	0 / 8	0.000
D	08/04/98	7026	Old	0.010	8 / 8	100.000
				0.014	8 / 8	100.000
				0.019	7 / 8	87.500
				0.027	7 / 8	87.500
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	08/04/98	7027	New	0.010	8 / 8	100.000
				0.014	7 / 8	87.500
				0.019	8 / 8	100.000
				0.027	8 / 8	100.000
				0.039	8 / 8	100.000
				0.055	0 / 8	0.000
D	08/05/98	7035	Old	0.010	7 / 8	87.500
				0.014	8 / 8	100.000
				0.019	8 / 8	100.000
				0.027	1 / 8	12.500
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	08/05/98	7036	New	0.010	8 / 8	100.000
				0.014	7 / 8	87.500
				0.019	8 / 8	100.000
				0.027	8 / 8	100.000
				0.039	8 / 8	100.000
				0.055	0 / 8	0.000
D	09/01/98	7128	Old	0.010	6 / 8	75.000
				0.014	8 / 8	100.000
				0.019	8 / 8	100.000
				0.027	0 / 8	0.000
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	09/01/98	7129	New	0.028	5 / 8	62.500
				0.039	7 / 8	87.500
				0.055	0 / 8	0.000
				0.078	0 / 8	0.000
				0.110	0 / 8	0.000
				0.160	0 / 8	0.000
D	09/03/98	7131	Old	0.010	6 / 8	75.000
				0.014	7 / 8	87.500
				0.019	7 / 8	87.500
				0.027	7 / 8	87.500
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	09/03/98	7132	New	0.028	7 / 8	87.500
				0.039	8 / 8	100.000

MREF Task 97-53

Table A-3. Listing of Experimental Data for Avidity Analysis

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
D	09/22/98	7172	Old	0.0011	0 / 6	0.0
				0.0015	0 / 6	0.0
				0.0022	0 / 6	0.0
				0.0031	0 / 6	0.0
				0.0044	0 / 6	0.0
				0.0062	1 / 6	16.7
D	09/22/98	7173	Old	0.00022	0 / 6	0.0
				0.00031	0 / 6	0.0
				0.00043	3 / 6	50.0
				0.00062	4 / 6	66.7
				0.00088	6 / 6	100.0
				0.0012	5 / 6	83.3

APPENDIX B:

CHARACTERIZATION OF SEROTYPE D ANTITOXIN

**[Excerpted from Battelle Memorial Institute
Medical Research and Evaluation Facility Biological Defense
Progress Report for JPO-BD Tasks, January 1999]**

MREF Task 97-53

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
D	09/03/98	7132	New	0.055	6 / 8	75.000
				0.078	0 / 8	0.000
				0.110	0 / 8	0.000
				0.160	0 / 8	0.000

Table A-2. Listing of Experimental Data for Serospecificity Analysis

Serotype	Experiment Date	Experiment Number	Group	Number Dead / Total	Pct. Dead
A	08/26/98	7090	A	0 / 4	0
			B	4 / 4	100
			C	2 / 4	50
			D	4 / 4	100
			E	3 / 4	75
C	08/26/98	7091	A	4 / 4	100
			B	4 / 4	100
			C	0 / 4	0
			D	4 / 4	100
			E	3 / 4	75
D	09/16/98	7163	A	4 / 4	100
			B	4 / 4	100
			C	4 / 4	100
			D	0 / 4	0
			E	4 / 4	100

Table A-3. Listing of Experimental Data for Avidity Analysis

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
A	09/10/98	7134	New	0.020	0 / 6	0.0
				0.028	0 / 6	0.0
				0.040	4 / 6	66.7
				0.057	6 / 6	100.0
				0.080	6 / 6	100.0
				0.11	6 / 6	100.0
A	09/10/98	7135	New	0.0020	0 / 6	0.0
				0.0028	0 / 6	0.0
				0.0040	0 / 6	0.0
				0.0057	0 / 6	0.0
				0.0080	6 / 6	100.0
				0.011	6 / 6	100.0
A	09/10/98	7136	New	0.00021	0 / 6	0.0
				0.00028	0 / 6	0.0
				0.00040	0 / 6	0.0
				0.00057	0 / 6	0.0
				0.00079	3 / 6	50.0
				0.0011	6 / 6	100.0
A	09/10/98	7138	Old	0.010	0 / 6	0.0
				0.014	0 / 6	0.0
				0.020	0 / 6	0.0
				0.028	2 / 6	33.3
				0.040	6 / 6	100.0
				0.057	6 / 6	100.0
A	09/10/98	7139	Old	0.0016	0 / 6	0.0
				0.0023	0 / 6	0.0
				0.0032	0 / 6	0.0
				0.0045	0 / 6	0.0
				0.0064	6 / 6	100.0
				0.0091	5 / 6	83.3
A	09/10/98	7140	Old	0.00013	0 / 6	0.0
				0.00019	0 / 6	0.0
				0.00026	0 / 6	0.0
				0.00038	0 / 6	0.0
				0.00053	1 / 6	16.7
				0.00075	4 / 6	66.7
C	09/09/98	7141	New	0.12	0 / 6	0.0
				0.17	0 / 6	0.0
				0.24	0 / 6	0.0
				0.34	6 / 6	100.0
				0.49	6 / 6	100.0
				0.69	6 / 6	100.0
C	09/09/98	7142	New	0.035	0 / 6	0.0
				0.049	0 / 6	0.0
				0.069	0 / 6	0.0
				0.098	6 / 6	100.0
				0.14	6 / 6	100.0
				0.20	5 / 6	83.3

MREF Task 97-53

Table A-3. Listing of Experimental Data for Avidity Analysis

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
C	09/09/98	7143	New	0.0053	0 / 6	0.0
				0.0075	2 / 6	33.3
				0.010	3 / 6	50.0
				0.015	6 / 6	100.0
				0.021	5 / 6	83.3
				0.029	6 / 6	100.0
C	09/09/98	7145	Old	0.24	0 / 6	0.0
				0.34	0 / 6	0.0
				0.49	1 / 6	16.7
				0.69	6 / 6	100.0
				0.97	6 / 6	100.0
				1.0	6 / 6	100.0
C	09/09/98	7146	Old	0.049	0 / 6	0.0
				0.069	0 / 6	0.0
				0.097	0 / 6	0.0
				0.14	6 / 6	100.0
				0.19	5 / 6	83.3
				0.27	6 / 6	100.0
C	09/09/98	7147	Old	0.0069	0 / 6	0.0
				0.0098	0 / 6	0.0
				0.014	3 / 6	50.0
				0.020	5 / 6	83.3
				0.028	6 / 6	100.0
				0.039	6 / 6	100.0
D	09/22/98	7168	New	0.014	0 / 6	0.0
				0.020	0 / 6	0.0
				0.028	0 / 6	0.0
				0.040	0 / 6	0.0
				0.057	1 / 6	16.7
				0.080	6 / 6	100.0
D	09/22/98	7169	New	0.0011	0 / 6	0.0
				0.0015	0 / 6	0.0
				0.0022	0 / 6	0.0
				0.0031	0 / 6	0.0
				0.0044	0 / 6	0.0
				0.0062	6 / 6	100.0
D	09/22/98	7170	New	0.00022	0 / 6	0.0
				0.00031	0 / 6	0.0
				0.00043	0 / 6	0.0
				0.00062	5 / 6	83.3
				0.00088	4 / 6	66.7
				0.0012	6 / 6	100.0
D	09/22/98	7171	Old	0.014	0 / 6	0.0
				0.020	0 / 6	0.0
				0.028	1 / 6	16.7
				0.040	0 / 6	0.0
				0.057	6 / 6	100.0
				0.080	5 / 6	83.3

APPENDIX B:

CHARACTERIZATION OF SEROTYPE D ANTITOXIN

[Excerpted from Battelle Memorial Institute Medical Research and Evaluation Facility Biological Defense Progress Report for JPO-BD Tasks, January 1999]

Comparison Studies With PerImmune Lyophilized (1998) and Liquid (1996) Antibody Standards for Serotypes A, C, and D

In July through September 1998, a series of studies were conducted by both PerImmune and Battelle to compare the relative potency of PerImmune lyophilized antibody standards (1998 issue) to the original liquid (1996 issue) standards. The 1998 product is a lyophilized version of the original 1996 issue. The standards were lyophilized in 1996 but not characterized and released to Battelle until 1998. Based on literature findings and prior experience with lyophilized antibodies, the expected recovery following lyophilization is in the range of 85-95 percent of the original activity. Both laboratories use the neutralization assay to measure antibody levels. Battelle uses the Cardella test levels (i.e., antibodies are titrated against approximately 3 LD₅₀ per mouse injection), whereas PerImmune uses somewhat higher test challenges. As the lyophilized standards are reconstituted in the same volume as the original standards, values are reported both in absolute terms and as a percent of the original activity found in the liquid standards (see below).

Serotype	PerImmune Results (n = 5)		Battelle MREF Results (n = 4)	
	Activity (U/mL)	Percent of Original Activity	Activity (U/mL)	Percent of Original Activity
A	77 ± 8	87 ± 10 (%)	106 ± 34	120 ± 39 (%)
C	38 ± 10	85 ± 23	35 ± 5	77 ± 12
D *	65 ± 13	113 ± 30	34 ± 5	60 ± 9

* Results were significantly different by t-test (P < 0.05).

The values obtained from both laboratories were not significantly different from each other nor different from the anticipated recovery for serotypes A and C. However, the Battelle value for serotype D was significantly lower than the PerImmune results as well as the expected recovery. To further explore the differences encountered for serotype D, both laboratories conducted additional potency tests. Results are presented below.

PerImmune Results

July 1998 (n=4)	113 ± 30 %
December 1998 (n=2)	93 ± 9 %
Overall average (n=6)	106 ± 26 %

Battelle Results

August 1998 (n=4, vials 98R-020 and 98R-025)	60 ± 9 %
December 1998 (n=3, vial 98R-021)	66 ± 2 %
(n=3, vial 98R-022)	60 ± 8 %
(n=3, vial 98R-196)	65 ± 7 %
December average of 3 vials	64 ± 6 %
Overall Average (n=13)	63 ± 7 %

Additional characterizations of preparations are given below.

1. Protein and UV Analyses

Sample	Protein (BCA assay)	UV (A280) 1/13/99	UV (A280) 1/21/99
PADT03/29/96 (liquid)	2.0 (units / ml)*	1.4 (mg/ml)	1.4
98R-021 (lyophilized)	2.0	1.1	Not run
98R-022 (lyophilized)	2.5	0.92	0.93
98R-196 (lyophilized)	1.6	0.56	0.66

* The protein values are normalized to a standard curve constructed with bovine serum albumin. One unit is equivalent to 1 mg BCA.

2. Fast Protein Liquid Chromatography (FPLC)

- Both 1996 and 1998 standards showed no evidence of protein breakdown. No other protein peaks identified other than monomeric immune globulin. The only other visible peaks were identified as lactose in the void volume (buffer is 4 percent lactose). See Examples 8, 9, and 10, below.

3. Stability Testing for Antibody Standards

- Initial testing 8/96 - Antibody standard characterization establishes that an average of 0.023 ± 0.008 U of antibody standard (serotype D) 50 percent neutralized 27 ± 2.3 MIPLD₅₀ units.
- Stability test 10/97 - 0.023 U of antibody standard 50 percent neutralized 35 ± 12 MIPLD₅₀ units. Value is not statistically higher than 8/96 benchmark value ($p > 0.05$).
- Stability test 12/98 - 0.023 U of antibody standard 50 percent neutralized 40.2 ± 10 MIPLD₅₀ units. Although higher than original value, the difference is not statistically significant from benchmark value ($p > 0.05$).
- In addition, control charts for neutralization assay standard curves were examined and there was evidence of change over the period of October 1997 (the previous stability test) to March 1998 (most recent use of antibody standard on study).

There is evidence that the potency of the 1996 in-house (Battelle) standard may be increasing over time. If true, the presumed cause would be evaporation. Given the imprecision of the neutralization assay, it is difficult to conclude this from the stability testing alone. UV absorption data, however, supports this conclusion. UV readings were higher for the 1996 in-house liquid standard than for the 1998 lyophilized standards and the original UV specification of 1 mg/ml reported by PerImmune in 1996. However, the BCA protein assay did not show a significant difference between the liquid and lyophilized standards. Based on discussions with PerImmune staff (Bill Rickman and Janet Ransom) and other resources, the

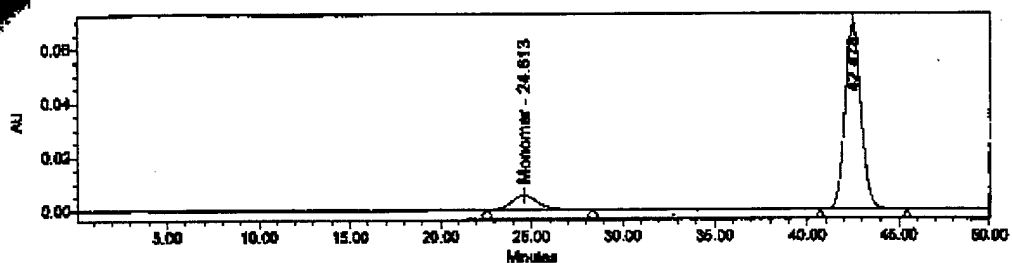
expected recovery of activity following the lyophilization should be around 90 percent. When PerImmune retested their standards in December 1998 they observed about a 10 percent loss of activity for all serotypes tested at that time.

CONCLUSIONS AND RECOMMENDATIONS

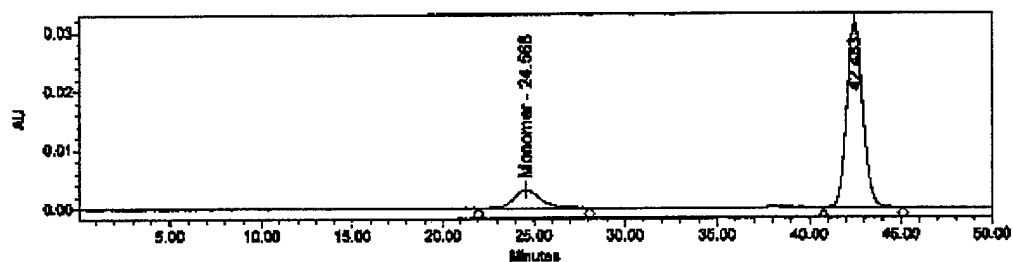
Given the internal consistency of PerImmune serotype D results with their other serotypes, a trend towards increasing potency during stability testing (Battelle), and an apparent increase in concentration as measured by UV absorption, we make the following recommendations:

1. As stability of the 1996 internal standard (Battelle) is not unequivocally demonstrated, these standards will no longer be used in-house (they have not been in use for the neutralization assay since March 1998).
2. The PerImmune calibration values were tested to identify the amount of toxin that is 50 percent neutralized by the standard antitoxin amount (0.023 U/ml). These values were in agreement with the original 1996 values (i.e., 27 MIPLD₅₀). The PerImmune units will be adopted for use on Task 161 and future tasks.

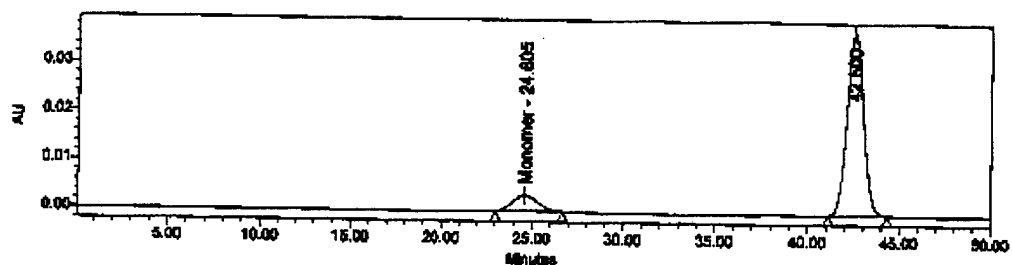
Example 8: Perimmune Antitoxin C, lot PACT 03/07/96



Example 9: Perimmune Antitoxin D, PADT 03/29/96 (liquid)



Example 10: Perimmune Antitoxin D, lot DAT 09/87 (lyoph)



Internal Distribution

Department Files
JR Holdcraft
MC Matthews*
NA Niemuth*
RMO

Date June 30, 1999

To Bob Hunt (JM-3)

From Jennifer Holdcraft *JRH*

* memo only

Subject **QA Materials for Task 53 Statistical
Report on Validation of Standards**

S:\Hold\BI3\Task53\Task53_QAMemo_StdValidation.doc

Overview

The SAS system (V6.12) was used to process and analyze data collected under MREF Task 97-53. Serospecificity, avidity, and head-to-head comparison experiments were summarized. For the head-to-head comparisons, probit models were fit to the antitoxin dose-lethality response data and used to estimate ED_{50} s for old and new standard preparations of botulinum serotypes A, C, and D. Paired t-tests compared the mean ED_{50} s for the old and new standards of each serotype. Finally, for each serotype, a likelihood ratio test (LRT) was used to determine whether the dose-response curves could be adequately described using a common slope and intercept model within each serotype and standard preparation, or if a common slope and separate intercept model would be needed.

Data Inputs/Outputs

Data were entered into a Microsoft Access (V97) database by MREF technicians. The data were transferred electronically to SAS datasets using the SAS/Access software, with an ODBC driver connection. The SAS program MOUSNEUT.SAS was used to read and merge data from the MS-Access tables. The SAS dataset MOUSNEUT.SD2 was created and used in the statistical analysis.

Performance Test Methods/Results

Performance testing for the transfer of data using the SAS program MOUSNEUT.SAS is provided separately.

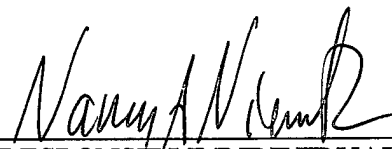
The SAS programs HEAD_TO_HEAD.SAS, SEROSPECIFICITY.SAS, and AVIDITY.SAS were used to process and analyze the data. A listing of each program and the output is attached.

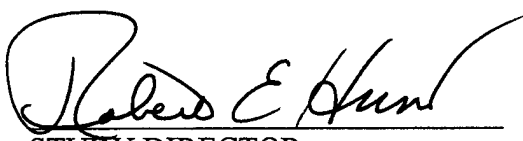
Probit models were fitted in the program HEAD_TO_HEAD.SAS and AVIDITY.SAS using the SAS PROBIT procedure and slope and intercept parameters were output directly to datasets. No performance tests of the PROBIT procedure calculations are required. ED_{50} s were calculated (this value cannot be output to a dataset automatically) and results were compared to probit output to verify the calculation.

Likelihood ratio tests (LRT) for each serotype were also performed in HEAD_TO_HEAD.SAS to determine the model (common slope, common intercept vs. common

slope, separate intercept) that would be needed. This test used parameters output from the PROBIT procedure and one value was calculated by hand to verify results.

Average potency values for Task 53 potency experiments were calculated and were used in the program AVIDITY.SAS to calculate toxin dose (MIP LD50 units/injection) for the avidity experiments. One toxin dose value was calculated by hand to verify the results. Avidity ratios were also computed in AVIDITY.SAS and results were verified by hand calculation for several values.


RESPONSIBLE INDIVIDUAL


STUDY DIRECTOR

JRH:llj
Attachment

For Review and Approval

	Name	Internal	Date
Originator	Jennifer Holdcraft	JRH	6/30/99
Concurrence	Nancy Niemuth	N	6/30/99
Approved	William Rosebrough	WRO	7/1/99

```

*****
* Name of Program      : head_to_head.sas
* Author              : Margaret Burke
*                    : Jennifer Holdcraft
* Date Created        : 10/9/98
*                    :
* Date Last Modified  : 6/28/99
*                    :
* Purpose of Program  : look at head to head comparisons of old and new
*                    : standards for task 53
*
* Data sets used      :
*
* Files/datasets produced :
*
* Note:               :
*****

```

Source code listing
of SAS program
head-to-head.sas
Jennifer R Holdcraft
6/29/99

```

libname saslib 'g:\projects\sas_mref\task53';

title 'MREF Task 97-53';

/* Use the dataset mousneut.sd2 that Claire Matthews provided
   only take the expnums for the head to head comparisons */

data mousneut;
  set saslib.mousneut (drop=indose outdose);
  where expnum in ('7013','7014','7022','7023','7031','7032','7040','7041',
    '7024','7025','7033','7034','7042','7043','7179','7180','7026','7027',
    '7035','7036','7128','7129','7131','7132');
  if dose < 0 then delete;
  if dose > 0 then logdose = log10(dose);
  label logdose = 'log10 concentration';
  if expnum in ('7013','7022','7031','7040','7024','7033','7042','7179','7026',
    '7035','7128','7131') then type='Old';
    else type='New';
  pct=pctdead*100;
run;

proc sort data=mousneut;
  by serotype expnum date descending type dose descending grpid;
run;

/* print out raw data listing for appendix of report and qa report */

options nodate nonumber;
title2 'Table A-1. Listing of Experimental Data for Head-to-Head Comparisons';
proc print data=mousneut label;
  label serotype='Serotype' date='Experiment Date' expnum='Experiment Number'
    type='Standard' dose='Dose (u/ml)' ondead='Number Dead / Total' pct='Pct. Dead';
  by serotype date expnum type;
  id serotype date expnum type;
  var dose ondead pct;
run;
options date number;

/* set indicator vars for whether responses span >50% to <50% */

data d1;
  set mousneut (drop=ondead pct);
  by serotype expnum date dose descending grpid;
  if first.expnum and pctdead gt .50 then gt50 = 1;
  if last.expnum and pctdead lt .50 then lt50 = 1;
run;

/* some overall stats for final table */

```

```

proc means noprint data=d1;
  where n > 0;
  by serotype expnum date type;
  var dose logdose ndead n norig nremove lt50 gt50;
  id assaytyp;
  output out=sumry1 sum=dum1 dum2 ndead n norig nremove lt50 gt50
         min= mindose logmin
         max= maxdose logmax
         n= ndoses;
run;

proc means noprint data=d1;
  where n > 0;
  by serotype;
  var logdose;
  output out=minmax (drop=_type_ _freq_)
         min=logmin
         max=logmax;
run;

data minmax;
  merge sumry1 (keep=serotype type expnum date) minmax;
  by serotype;
run;

/* fake records to get predicted values for smooth curve for plot */

data predrecs;
  set minmax;
  ipred=1;
  tempmin = round(logmin, .1);
  tempmax = round(logmax, .1);
  if serotype='A' then tempmin = round(log10(.005), .1);
  if serotype='A' then tempmax = round(log10(.1), .1);

  if logmin < tempmin then logmin = tempmin - .1;
  else logmin = tempmin;
  if logmax > tempmax then logmax = tempmax + .1;
  else logmax = tempmax;

  step = (logmax-logmin)/100;

  do logdose=logmin to logmax by step;
    dose = 10**logdose;
    output;
  end;
  drop logmin logmax step tempmin tempmax;
run;

proc sort data=d1; by serotype date expnum; run;

data d2;
  set d1 (in=in1) predrecs;
  if in1 and n le 0 then delete;

  ind1=0; ind2=0; ind3=0; ind4=0;

  if serotype='A' and date = '03aug98'd then ind1 = 1;
  else if serotype='A' and date = '04aug98'd then ind2 = 1;
  else if serotype='A' and date = '05aug98'd then ind3 = 1;
  else if serotype='A' then ind4 = 1;
  if serotype in ('C','D') and date = '04aug98'd then ind1 = 1;
  else if serotype in ('C','D') and date = '05aug98'd then ind2 = 1;
  else if serotype in ('C','D') and date in ('08aug98'd,'01sep98'd) then ind3 = 1;
  else if serotype in ('C','D') then ind4 = 1;

  pctdead = ndead/n;

```

```

run;

*** Common Slope/Common Intercept Model ***;

proc sort data=d2; by serotype; run;

proc probit data=d2 log10 hprob=0.01 outest=lcicso noprint; *lackfit;
  where type='Old';
  by serotype;
  model ndead/n = dose /d=normal inversecl;
  output out=predout p=predpct;
  label expnum = 'Experiment Number';
  label stdcurv = 'Corresponding Std. Curve';
  label serotype = 'Serotype';
run;

data edcco;
  set lcicso;
  type='Old';
  ed50=10**(-intercep/dose);
  keep serotype dose intercep ed50 _lnlike_ type;
run;

title2 "B1-3 Mouse Neutralization Studies";
title3 'Probit Results For Proportions Of Mice Dead Versus Log10 Antibody Concentration';
title4 'Old Standard - Common Slope/Common Intercept Model';
proc print data=edcco label;
  label serotype='Serotype' type='Standard' dose='Slope' intercep='Intercept'
    _lnlike_='Log Likelihood' ed50='ED50';
  var serotype type dose intercep _lnlike_ ed50;
run;

*** Common Slope/Separate Intercept Model ***;

proc probit data=d2 log10 hprob=0.01 outest=lsicso noprint; *lackfit;
  where type='Old';
  by serotype;
  model ndead/n = dose ind1 ind2 ind3 ind4 /d=normal inversecl noint;
  output out=predout p=predpct;
  label expnum = 'Experiment Number';
  label stdcurv = 'Corresponding Std. Curve';
  label serotype = 'Serotype';
run;

data edcso;
  set lsicso;
  type='Old';
  ed50=10**(-ind1/dose); intercep=ind1; exp=1; output;
  ed50=10**(-ind2/dose); intercep=ind2; exp=2; output;
  ed50=10**(-ind3/dose); intercep=ind3; exp=3; output;
  ed50=10**(-ind4/dose); intercep=ind4; exp=4; output;
  keep serotype dose intercep exp ed50 _lnlike_ type;
run;

title2 "B1-3 Mouse Neutralization Studies";
title3 'Probit Results For Proportions Of Mice Dead Versus Experiment Day And Log10 Antibody Concentration';
title4 'Old Standard - Common Slope/Separate Intercept Model';
proc print data=edcso label;
  label serotype='Serotype' type='Standard' dose='Slope' intercep='Intercept'
    _lnlike_='Log Likelihood' ed50='ED50';
  var serotype type dose intercep _lnlike_ ed50;
run;

*** For New Standards ***;
*** Common Slope/Common Intercept Model ***;

proc probit data=d2 log10 hprob=0.01 outest=lcicso noprint; *lackfit;

```

ED50 calculation $ED_{50} = 10^{\left(\frac{-intercept}{slope}\right)}$

```

where type='New';
by serotype;
model ndead/n = dose /d=normal inversecl;
output out=predout p=predpct;
label expnum = 'Experiment Number';
label stdcurv = 'Corresponding Std. Curve';
label serotype = 'Serotype';
run;

data edcon;
set lcicsn;
type='New';
ed50=10**(-intercep/dose);
keep serotype dose intercep ed50 _lnlike_ type;
run;

title2 'B1-3 Mouse Neutralization Studies';
title3 'Probit Results For Proportions Of Mice Dead Versus Log10 Antibody Concentration';
title4 'New Standard - Common Slope/Common Intercept Model';
proc print data=edcon label;
  label serotype='Serotype' type='Standard' dose='Slope' intercep='Intercept'
    _lnlike_='Log Likelihood' ed50='ED50';
  var serotype type dose intercep _lnlike_ ed50;
run;

*** Common Slope/Separate Intercept Model ***;

proc probit data=d2 log10 hprob=0.01 outest=lsicsn noprint; *lackfit;
  where type='New';
  by serotype;
  model ndead/n = dose ind1 ind2 ind3 ind4 /d=normal inversecl noint;
  output out=predout p=predpct;
  label expnum = 'Experiment Number';
  label stdcurv = 'Corresponding Std. Curve';
  label serotype = 'Serotype';
run;

data edcsn;
set lsicsn;
type='New';
ed50=10**(-ind1/dose); intercep=ind1; exp=1; output;
ed50=10**(-ind2/dose); intercep=ind2; exp=2; output;
ed50=10**(-ind3/dose); intercep=ind3; exp=3; output;
ed50=10**(-ind4/dose); intercep=ind4; exp=4; output;
keep serotype dose intercep exp ed50 _lnlike_ type;
run;

title2 'B1-3 Mouse Neutralization Studies';
title3 'Probit Results For Proportions Of Mice Dead Versus Experiment Day And Log10 Antibody Concentration';
title4 'New Standard - Common Slope/Separate Intercept Model';
proc print data=edcsn label;
  label serotype='Serotype' type='Standard' dose='Slope' intercep='Intercept'
    _lnlike_='Log Likelihood' ed50='ED50';
  var serotype type dose intercep _lnlike_ ed50;
run;

/* put together table of old and new results for common slope and intercept model */

data ccmodel;
set edcco edcon;
keep serotype type ed50;
run;

proc sort data=ccmodel; by serotype descending type; run;

title2 'ED50s from Common Slope and Intercept Model For Each Serotype and Type';
proc print data=ccmodel label;

```


B1-3 Mouse Neutralization Studies

Probit Results For Proportions Of Mice Dead Versus Log10 Antibody Concentration
New Standard - Common Slope/Common Intercept Model

OBS	Serotype	Standard	Slope	Intercept	Log Likelihood	ED50
1	A	New	-5.42470	-9.31603	-68.2819	0.019172
2	C	New	-3.85482	-6.77505	① -82.9972	0.017476
3	D	New	-5.42895	-7.28471	-57.7486	0.045517

Probit output directly to dataset for 1998 (New)
Standard - Common Slope & Intercept Model

Jennifer R Holdcraft
6/28/99

B1-3 Mouse Neutralization Studies

Probit Results For Proportions Of Mice Dead Versus Experiment Day And Log10 Antibody Concentration
New Standard - Common Slope/Separate Intercept Model

OBS	Serotype	Standard	Slope	Intercept	Log Likelihood	ED50
1	A	New	-6.91482	-12.9757	-53.5131	0.013289
2	A	New	-6.91482	-11.4546	-53.5131	0.022053
3	A	New	-6.91482	-12.1064	-53.5131	0.017750
4	A	New	-6.91482	-10.9179	-53.5131	0.026368
5	C	New	-3.98837	-6.8565	-79.8471	0.019093
6	C	New	-3.98837	-6.7189	-79.8471	0.020672
7	C	New	-3.98837	-7.4665	-79.8471	0.013426
8	C	New	-3.98837	-6.9757	-79.8471	0.017823
9	D	New	-5.83680	-7.9215	-53.9135	0.043937
10	D	New	-5.83680	-7.9215	-53.9135	0.043937
11	D	New	-5.83680	-8.3057	-53.9135	0.037757
12	D	New	-5.83680	-7.2865	-53.9135	0.056446

Probit results output directly to dataset for 1998 (New) Standards for Common Slope, Separate Intercept Model. Compare these results to Table 1 in "Validation of Antitoxin Standards.. " report (New standard rows only). Data sorted by date & expnum.

Jennifer R. Holdcraft
6/28/99

Log Likelihood Ratio Test for Differences Between Dates Among Serotypes
 Comparison of Common Slope and Intercept-New Model vs. Common Slope and Separate Intercept-New Model

OBS	Serotype	Common Slope and Intercept-New	Common Slope and Separate Intercept-New	Likelihood Ratio Test	P-Value
1	A	-68.2819	-53.5131	29.5376	<u>0.000000</u> → < 0.001
* 2	C	① -82.9972	② -79.8471	6.3002	<u>0.012072</u>
3	D	-57.7486	-53.9135	7.6702	<u>0.005614</u> → 0.006

Log Likelihood Test for Day to Day Variability
 (Common Slope & Intercept vs. Common Slope / Separate Int.)
 in 1998 (New) Standard. All serotypes
 significant - again, support for adoption of
 common slope, separate intercept model. Serotype
 A and D p-values given in 1st paragraph
 of Results section of report.

Jennifer R Holdcraft
 6/29/99

* compare log likelihood values to those on previous
 2 pages

Avidity Measurements - Number of MIPLD50 Units per Injection at Test Level

Serotype	Year	Standard	L+/10	L+/33	L+/100
A	1996	Old	1031.51	211.182	24.3483
	1998	New	1414.34	243.677	28.5126
C	1996	Old	1225.75	303.656	35.9451
	1998	New	685.70	218.062	23.7760
D	1996	Old	1852.16	253.578	20.5233
	1998	New	2299.56	202.236	24.3400

ED₅₀'s from previous page arranged according to whether expnuu was an avidity 10, 33, or 100 experiment.

These match Table 4a of report (excluding Task 39 column).

Jennifer R Holdcraft
6/28/99

AVIDITY
RESULTS

OBS	Serotype	Experiment Number	Slope	Intercept	Calculated ED50
1	A	7134	48.320	-152.235	1414.34
2	A	7135	114.490	-273.267	243.68
3	A	7136	49.810	-72.475	28.51
4	A	7138	48.352	-145.707	1031.51
5	A	7139	9.417	-21.891	211.18
6	A	7140	10.000	-13.865	24.35
7	C	7141	111.762	-316.971	685.70
8	C	7142	6.473	-15.139	218.06
9	C	7143	5.296	-7.288	23.78
10	C	7145	54.003	-166.783	1225.75
11	C	7146	10.223	-25.377	303.66
* 12	C	7147	9.764	-15.190	35.95 ✓
13	D	7168	54.898	-184.547	2299.56
14	D	7169	114.149	-263.212	202.24
15	D	7170	7.478	-10.367	24.34
16	D	7171	6.386	-20.867	1852.16
17	D	7172	41.040	-98.665	253.58
18	D	7173	5.093	-6.683	20.52

Probit results output to dataset with
calculated ED50. Verify calculation
* by hand... $ED_{50} = 10^{\left(\frac{-\text{intercept}}{\text{slope}}\right)}$
 $= 10^{\left(\frac{-(-15.190)}{9.764}\right)}$
 $= 10^{\left(\frac{1.554548355}{1.555714871}\right)}$
 $= 35.9513225 \approx 35.95 \checkmark$

Jennifer R Holdcraft
6/28/99

Paired T-Test Results Comparing Average ED50 for Old and New Standards of Each Serotype

OBS	Serotype	Average ED50 - Old Standard	Std. Dev. ED50 - Old Standard	Average ED50 - New Standard	Std. Dev. ED50 - New Standard	T Statistic	P-Value
1	A	0.025857	.0045780	0.019865	.0056214	-1.99501	0.14000
2	C	0.015898	.0005208	0.017754	.0031115	1.31348	0.28044
3	D	0.024200	.0040082	0.045520	.0078453	5.53017	0.01165

Proc Means Results calculating mean and standard deviation for each serotype and standard, and proc means t-test and p-value result comparing 1996 (old) and 1998 (new) standards within each serotype. Compare results to those in Table 2 of Validation of Antitoxin Standards report.

6/29/99 Jennifer R Holdcraft

Serotype	Year	Standard	$\frac{[L+/10]}{[3.3 \cdot L+/33]}$	$\frac{[L+/33]}{[3 \cdot L+/100]}$	$\frac{[L+/10]}{[10 \cdot L+/100]}$
A	1996	Old	1.48014	2.89113	4.23649
	1998	New	1.75884	2.84877	4.96042
* C →	1996	Old	1.22322 ✓	2.81592 ✓	3.41006 ✓
	1998	New	0.95288	3.05716	2.88398
D	1996	Old	2.21336	4.11854	9.02463
	1998	New	3.44566	2.76959	9.44766

↑
Ratio of
 $\frac{\text{Avid 10}}{3.3(\text{Avid 33})}$

↑
Ratio of
 $\frac{\text{Avid 33}}{3(\text{Avid 100})}$

↑
Ratio of
 $\frac{\text{Avid 10}}{10(\text{Avid 100})}$

Check Avidity Ratios by Hand for one case...

C 1996 Old $L+/10 = 1225.75$
 $L+/33 = 303.656$
 $L+/100 = 35.9451$

$$\frac{L+/10}{3.3(L+/33)} = \frac{1225.75}{3.3(303.656)} = 1.223224 \approx 1.22322 \checkmark$$

$$\frac{L+/33}{3(L+/100)} = \frac{303.656}{3(35.9451)} = 2.815923 \approx 2.81592 \checkmark$$

$$\frac{L+/10}{10(L+/100)} = \frac{1225.75}{10(35.9451)} = 3.410061 \approx 3.41006 \checkmark$$

These values match those in Table 4b
of report (excluding Task 39 column)

6/29/99 Jennifer R Holdcraft

```

label serotype='Serotype' type='Standard Type' ed50='ED50';
var serotype type ed50;
run;

/* put together table of old and new results for common slope, separate intercept
model, and get average ed50 for each serotype */

proc sort data=d2; by serotype type expnum; run;

data expnum;
set d2;
by serotype type expnum;
if first.expnum;
keep serotype type expnum date;
run;

data csmodel;
set edcso edcsn;
keep serotype type exp ed50 dose intercep;
run;

proc sort data=csmodel; by serotype type exp; run;

proc means data=csmodel mean noprint;
by serotype type;
var ed50;
output out=means (drop=_type_ _freq_)
mean=avged50;
run;

data csmodel;
merge csmodel means expnum;
by serotype type;
run;

proc sort data=csmodel; by serotype descending type expnum; run;

title2 'ED50s from Common Slope and Separate Intercept Model For Each Serotype and Type';
proc print data=csmodel label;
label serotype='Serotype' type='Standard Type' expnum='Experiment Number' ed50='ED50'
avged50='Average ED50' date='Experiment Date' dose='Slope' intercep='Intercept';
var serotype date expnum type dose intercep ed50;
run;

data _null_;
set csmodel;
file 'd:/my projects/mref/task53/results/ed50.txt';
if _n_=1 then put 'Serotype $ Date $ Experiment Number $ Standard $ Slope $ Intercept $ ED50';
put serotype '$' date '$' expnum '$' type '$' dose 5.2 '$' intercep 5.2 '$' ed50 5.3;
run;

/* code for likelihood ratio test of differences between models */

%macro likratio (data1,data2,df,modname1,modname2);

data &data1.1;
set &data1;
keep _lnlike_ serotype;
where _type_='PARMS';
rename _lnlike_=_&data1._;
run;

proc sort data=&data1.1;
by serotype;
run;

```



```

data &data2.1;
  set &data2;
  keep _lnlike_ serotype;
  where _type_='PARMS';
  rename _lnlike_ = &data2._;
run;

proc sort data=&data2.1;
  by serotype;
run;

data calc;
  merge &data1.1
        &data2.1;
  by serotype;
  lrt=abs(-2*( _&data1._ - &data2._ ));
  p_value=1-probchi(lrt,&df);
run;

title2 'Log Likelihood Ratio Test for Differences Between Dates Among Serotypes';
title3 'Comparison of &Modname1 Model vs. &Modname2 Model';

proc print data=calc label;
  label _&data1._ = "&Modname1" _&data2._ = "&Modname2" serotype = 'Serotype' p_value = 'P-Value'
        lrt = 'Likelihood Ratio Test';
  var serotype _&data1._ _&data2._ lrt p_value;
run;

%mend likratio;

%likratio (lcicso,lsicso,1,Common Slope and Intercept-Old,Common Slope and Separate Intercept-Old)
%likratio (lcicsn,lsicsn,1,Common Slope and Intercept-New,Common Slope and Separate Intercept-New)

*** Compare Old and New Standards ***;

data d3; set d2;
  indold = 0;
  indnew = 0;
  if type='Old' then indold = 1;
  else indnew = 1;
  indold1 = ind1*indold;
  indold2 = ind2*indold;
  indold3 = ind3*indold;
  indold4 = ind4*indold;
  indnew1 = ind1*indnew;
  indnew2 = ind2*indnew;
  indnew3 = ind3*indnew;
  indnew4 = ind4*indnew;
run;

proc sort data=d3;
  by serotype expnum stdcurv dose;
run;

*** common slope/common intercept model for old and new standard ***;

proc probit data=d3 logit hprob=0.01 outest=lcicsa noprint; *lackfit;
  by serotype;
  model ndead/n = dose /d=normal inversed1;
  output out=predout p=predpct;
  label expnum = 'Experiment Number';
  label stdcurv = 'Corresponding Std. Curve';
  label serotype = 'Serotype';
run;

data edcca;

```

```

set lsicsa;
type='All';
ed50=10**(-intercep/dose);
keep serotype dose intercep ed50 _lnlike_ type;
run;

title2 'B1-3 Mouse Neutralization Studies';
title3 'Probit Results For Proportions Of Mice Dead Versus Experiment Day And Log10 Antibody Concentration';
title4 'Old And New Standard - Common Slope/Common Intercept Model';
proc print data=edoca label;
  label serotype='Serotype' type='Standard' dose='Slope' intercep='Intercept'
        _lnlike_='Log Likelihood' ed50='ED50';
  var serotype type dose intercep _lnlike_ ed50;
run;

*** common slope/separate intercept model for old and new standard ***;

proc probit data=d3 log10 hprob=0.01 outest=lsicsa noprint; *lackfit;
  by serotype;
  model ndead/n = dose indold1 indold2 indold3 indold4 indnew1
                 indnew2 indnew3 indnew4 /d=normal inversecl noint;
  output out=predfml p=predpct;
  label expnum = 'Experiment Number';
  label stdcurv = 'Corresponding Std. Curve';
  label serotype = 'Serotype';
run;

data edoca;
  set lsicsa;
  type='Old';
  ed50=10**(-indold1/dose); intercep=indold1; exp=1; output;
  ed50=10**(-indold2/dose); intercep=indold2; exp=2; output;
  ed50=10**(-indold3/dose); intercep=indold3; exp=3; output;
  ed50=10**(-indold4/dose); intercep=indold4; exp=4; output;
  type='New';
  ed50=10**(-indnew1/dose); intercep=indnew1; exp=1; output;
  ed50=10**(-indnew2/dose); intercep=indnew2; exp=2; output;
  ed50=10**(-indnew3/dose); intercep=indnew3; exp=3; output;
  ed50=10**(-indnew4/dose); intercep=indnew4; exp=4; output;
  keep serotype dose intercep ed50 _lnlike_ type;
run;

title2 'B1-3 Mouse Neutralization Studies';
title3 'Probit Results For Proportions Of Mice Dead Versus Experiment Day And Log10 Antibody Concentration';
title4 'Old And New Standard - Common Slope/Separate Intercept Model';
proc print data=edoca label;
  label serotype='Serotype' type='Standard' dose='Slope' intercep='Intercept'
        _lnlike_='Log Likelihood' ed50='ED50';
  var serotype type dose intercep _lnlike_ ed50;
run;

/* run likelihood ratio test macro */

%likratio (lcicsa,lsicsa,7,Common Slope and Intercept-Old and New,Common Slope and Separate Intercept-Old and New);

*****;
*** Test for Significant Differences in Old ED50 average and New ED50 average ***;

data old (drop=type avged50);
  set csmodel;
  where type='Old';
  rename ed50=olded50 expnum=oldexp;
run;

data new (drop=type avged50);

```

```

set csmodel;
where type='New';
rename ed50=newed50 expnum=newexp;
run;

data paired;
merge old new;
by serotype exp;
diff=newed50-olded50;
run;

proc sort data=paired;
by serotype;
run;

proc means data=paired mean std t prt noprint;
by serotype;
var olded50 newed50 diff;
output out=summary (drop= _type_ _freq_ junk1 junk2)
mean=oldmean newmean
std=oldstd newstd
t=junk junk0 tval
prt=junk1 junk2 pval;
run;

title2 'Paired T-Test Results Comparing Average ED50 for Old and New Standards of Each Serotype';
proc print data=summary label;
label serotype='Serotype' oldmean='Average ED50 - Old Standard'
oldstd='Std. Dev. ED50 - Old Standard' newmean='Average ED50 - New Standard'
newstd='Std. Dev. ED50 - New Standard' tval='T Statistic' pval='P-Value';
var serotype oldmean oldstd newmean newstd tval pval;
run;

data _null_;
set summary;
file 'd:/my projects/mraf/task53/results/t_test.txt';
if _n_=1 then put 'Serotype $ Old - Mean $ Old - Std $ New - Mean $ New - Std $ P-value';
put serotype '$' oldmean 5.3 '$' oldstd 5.3 '$' newmean 5.3 '$' newstd 5.3 '$' pval 5.3;
run;

*****;
*** Make cgm graphs for each serotype - these are graphs for the report ***;

data predsym;
set predfn1 csmodel (in=inmod rename=(ed50=dose) keep=serotype ed50 type date exp);
if inmod then predpct=0.5;
run;

proc sort data=predsym; by serotype descending type date descending predpct; run;

data plot;
set predsym;
by serotype descending type date;
retain code;
predpct=predpct*100;
if type='Old' then code=1;
else code=2;
if predpct=50 then code=2+exp;
output;
if last.date then do;
predpct=.;
output;
end;
/* repeat last predicted pt of each serotype/date combo as missing so skipmiss option
keeps sas from drawing line from end of one graph to beginning of another */
run;

```

```

proc sort data=plot; by serotype descending type date descending predpct; run;

proc greplay nofs igout=gsag;
  delete _all_;
  run; quit;

%macro makecgm(sero);

GOPTIONS reset=goptions DEVICE=cgmwwc ftext=hwcm001 htext=1.5
  nopolygonfill rotate=landscape NOPROMPT GSFMODE=REPLACE GSFNAME=GRAFOUT;

axis1 label=('Dose (u/ml)')
  logbase=10 logstyle=expand;

axis2 label=(a=90 r=0 'Percent Lethality (%)') minor=none;

symbol1 value=none i=join l=1 color=black;
symbol2 value=none i=join l=2 w=8 color=black;
symbol3 value=star h=1.3 i=none color=black;
symbol4 value=circle h=1.7 i=none color=black;
symbol5 value=diamond h=1.5 i=none color=black;
symbol6 value=square h=1.5 i=none color=black;

legend2 label=none
  value = (H=1.3
    t=1 j=1 ' Old Standard'
    t=2 j=1 ' New Standard'
    t=3 j=1 ' Exp 1'
    t=4 j=1 ' Exp 2'
    t=5 j=1 ' Exp 3'
    t=6 j=1 ' Exp 4')
  offset=(2,0)
  frame
  across=2 down=3;

FILENAME GRAFOUT 'd:\my projects\mref\task53\results\sero_&sero..cgm';

title;
proc gplot data=plot nocache;
  where serotype='&sero';
  plot predpct*dose=code/haxis=axis1 vaxis=axis2 legend=legend2 frame skipmiss;
run;
quit;

%mend makecgm;

%makecgm(A);
%makecgm(C);
%makecgm(D);

*****;
*** Make simple graphs to check model fit for each serotype ***;

proc sort data=predfn1; by serotype descending type date; run;

data simple;
  set predfn1;
  if pctdead=. then code=1;
  else code=2;
  predpct=predpct*100;
  if pctdead ne . then predpct=pctdead*100;
run;

GOPTIONS reset=goptions reset=all DEVICE=win targetdevice=winprtm ftext=duplex
  htext=1.5 nopolygonfill noprompt display;

axis1 label=('Dose (u/ml)')

```

```

logbase=10 logstyle=expand;

axis2 label=(a=90 r=0 'Percent Lethality (%)') minor=none;

symbol1 value=none i=join l=1 color=black;
symbol2 value=star h=1.3 i=none color=black;

legend2 label=none
value = (H=1.3
        t=1 j=1 ' Probit Model'
        t=2 j=1 ' Actual Data ')
offset=(2,0)
frame
across=2 down=1;

proc greplay nofs igout=gseg;
delete _all_;
run; quit;

title;
proc gplot data=simple nocache;
by serotype descending type date;
plot predpct*dose=code/haxis=axis1 vaxis=axis2 legend=legend2 frame skipmiss;
run;
quit;

filename job 'c:\sas help';
%include job(tgraf8);

proc greplay nofs tc=tcatt igout=gseg;
template graf8;
tplay 1:gplot 2:gplot1 3:gplot2 4:gplot3 5:gplot4 6:gplot5 7:gplot6 8:gplot7;
run; quit;

proc greplay nofs tc=tcatt igout=gseg;
template graf8;
tplay 1:gplot8 2:gplot9 3:gplot10 4:gplot11 5:gplot12 6:gplot13 7:gplot14 8:gplot15;
run; quit;

proc greplay nofs tc=tcatt igout=gseg;
template graf8;
tplay 1:gplot16 2:gplot17 3:gplot18 4:gplot19 5:gplot20 6:gplot21 7:gplot22 8:gplot23;
run; quit;

```

```

/*****
**** Program Name: serospecificity.sas
**** Purpose: perform serospecificity analysis
**** Project Number:
**** Date Written: 9/18/98
**** Last Update: 6/29/99
**** Data Sets Used:
**** Data Sets Created:
**** Files Used:
**** Files Created:
**** Comments:
**** Programmer: Jennifer R. Holdcraft
*****/

```

Source code listing of
Sas program SEROSPECIFICITY.SAS
Jennifer R Holdcraft
6/29/99

```
libname saslib 'g:\projects\sas_mref\task53';
```

```
title 'MREF Task 97-53';
```

```

data sero;
  set saslib.mousneut;
  where expnum in ('7090','7091','7163') and grpid ne 'F';
  pct=pctdead*100;
  if serotype='A' and grpid ne 'A' then toxlvl=50;
  else if serotype='A' then toxlvl=1000;
  else if serotype='C' and grpid not in ('C','D') then toxlvl=100;
  else if serotype='C' and grpid='C' then toxlvl=2000;
  else if serotype='C' and grpid='D' then toxlvl=1000;
  else if serotype='D' and grpid not in ('C','D') then toxlvl=50;
  else if serotype='D' and grpid='D' then toxlvl=1000;
  else if serotype='D' and grpid='C' then toxlvl=500;
  pctsurv=100-pct;
  onsurv=4-substr(left(ondead),1,1)||substr(ondead,3);
  keep serotype date expnum ondead grpid pctdead pct toxlvl pctsurv onsurv;
run;

```

(%)
← pct survived = 100 - pct dead
← #survived = 4 - #dead

↓ gives #survived/total column

```

proc sort data=sero;
  by serotype date expnum grpid;
run;

```

```

options nodate nonumber;
title2 'Table A-2. Listing of Experimental Data for Serospecificity Analysis';
proc print data=sero label;
  label serotype='Serotype' date='Experiment Date' expnum='Experiment Number'
  grpid='Group' ondead='Number Dead / Total' pct='Pct. Dead';
  by serotype date expnum;
  id serotype date expnum;
  var grpid ondead pct;
run;
options date number;

```

```

title2 'Summary of Serospecificity Experiments for 1998 Standards';
proc print data=sero label;
  label serotype='Antitoxin Standard' date='Experiment Date' expnum='Experiment Number'
  grpid='Botulinum Serotype' toxlvl='Toxin Dose' ondead='Number Dead / Total'
  pct='Pct. Dead' onsurv='Number Survived / Total' pctsurv='Pct. Survived';
  by serotype date expnum;
  id serotype date expnum;
  var grpid ondead pct toxlvl onsurv pctsurv;
run;

```

```

/*****
**** Program Name: Avidity.sas
**** Purpose: perform avidity analysis
**** Project Number:
**** Date Written: 9/18/98
**** Last Update: 6/28/99
**** Data Sets Used:
**** Data Sets Created:
**** Files Used:
**** Files Created:
**** Comments:
**** Programmer: Jennifer R. Holdcraft
*****/

```

Source code listing of
SAS program AVIDITY.SAS
Jennifer R Holdcraft
6/29/99

```

libname saslib 'd:\my projects\mref\task53\sas datasets';

title 'MREF Task 97-53';

proc sql feedback;
connect to odbc as task53('DSN=MS Access 97 Database;DBQ=\\Ns-bwj-fs1\mrefprj\Task97-53\database\Task53_be.mdb;
UID=holdcraft;PWD=mrefpw;SystemDB=\\Ns-bwj-fs1\mrefprj\AccessWkGrp\systemmref.mdw;');

```

```

CREATE TABLE av AS (select *
from connection to task53 (select studydate as sdatt,
ExpNum,
Group,
NumInGrp as n,
TestSerumAmt as dose,
NumInGrp-Survival as ndead,
((NumInGrp-Survival)/NumInGrp)*100 as pctdead
from tblNAAssay ));

```

```

disconnect from task53;
quit;

```

```

data avid;
set av;
where expnum in ('7134','7135','7136','7138','7139','7140','7141','7142','7143',
'7145','7146','7147','7168','7169','7170','7171','7172','7173')
and group not in ('H','I','G');
expnum=expnum*1;
if expnum<=7140 then do;

```

```

serotype='A';
potency=144387.3907;

```

```

end;
else if expnum<=7147 then do;

```

```

serotype='C';
potency=9601.772511;

```

```

end;

```

```

else do;

```

```

serotype='D';
potency=154955.86102;

```

```

end;

```

```

ld50unit=(dose*potency)/4;

```

```

if expnum in (7134, 7135, 7136, 7141, 7142, 7143, 7168, 7169, 7170) then year=1998;

```

```

else year=1996;

```

```

if year=1998 then standard='New';

```

```

else standard='Old';

```

```

ondead=ndead||' / '||trim(left(n));

```

```

date=datepart(sdatt);

```

```

format date mmddyy8.;

```

```

drop sdatt;

```

```

run;

```

```

proc sort data=avid;
by serotype date expnum standard dose;

```

← hand entered average
potencies. can check
to see that they
match following pages
qkt

← toxin dose calculation

```

;
run; */

title2 'Avidity Measurements - Number of MIPLD50 Units per Injection at Test Level';
proc print data=unitneut label;
  label serotype='Serotype' year='Year' std='Standard' lev10='L+/10'
    lev33='L+/33' lev100='L+/100';
  by serotype;
  id serotype;
  var year std lev10 lev33 lev100;
run;

data ratio;
  set unitneut;
  rat1033=lev10/(3.3*lev33);
  rat33100=lev33/(3*lev100);
  rat10100=lev10/(10*lev100);
run;

title2 'Avidity Ratios';
proc print data=ratio label;
  label serotype='Serotype' year='Year' std='Standard' rat1033='[L+/10] / [3.3*L+/33]'
    rat33100='[L+/33] / [3*L+/100]' rat10100='[L+/10] / [10*L+/100]';
  by serotype;
  id serotype;
  var year std rat1033 rat33100 rat10100;
run;

```



```

run;

options nodate nonumber;
title2 'Table A-3. Listing of Experimental Data for Avidity Analysis';
proc print data=avid label;
  label serotype='Serotype' date='Experiment Date' expnum='Experiment Number'
        standard='Standard' dose='Dose (u/ml)' ondead='Number Dead / Total'
        pctdead='Pct. Dead' ld50unit='Toxin Dose (MIP LD50 units/injection)';
  by serotype date expnum standard;
  id serotype date expnum standard;
  var dose ld50unit ondead pctdead;
  format pctdead 5.1;
run;
title2;
options date number;

proc probit data=avid log10 outest=ests noprint;
  by serotype expnum;
  model ndead/n = ld50unit/d=normal inversecl;
  output out=proout P=PREDPCT;
run;

data ests2;
  set ests;
  pct50=10**(-intercep/ld50unit);
run;

title2 'Probit Results For Avidity Experiments';
proc print data=ests2 label;
  label serotype='Serotype' expnum='Experiment Number' ld50unit='Slope'
        intercep='Intercept' pct50='Calculated ED50';
  var serotype expnum ld50unit intercep pct50;
run;

data lev10 lev33 lev100;
  set ests;
  pct50=10**(-intercep/ld50unit);
  if expnum in (7134, 7135, 7136, 7141, 7142, 7143, 7168, 7169, 7170) then year=1998;
  else year=1996;
  if year=1998 then std='New';
  else std='Old';
  if expnum in (7134,7138,7141,7145,7168,7171) then output lev10;
  else if expnum in (7135,7139,7142,7146,7169,7172) then output lev33;
  else output lev100;
  keep serotype year std pct50;
run;

proc sort data=lev10; by serotype year std; run;
proc sort data=lev33; by serotype year std; run;
proc sort data=lev100; by serotype year std; run;

data unitneut;
  merge lev10 (rename=(pct50=lev10)) lev33 (rename=(pct50=lev33)) lev100 (rename=(pct50=lev100));
  by serotype year std;
run;

/* old code from when entered 50th pctile by hand - not needed anymore

data unitneut;
  input serotype$ year std$ lev10 lev33 lev100;
  cards;
A 1998 New 1414.34411 243.67687 28.51256
A 1996 Old 1031.51446 211.18218 24.34832
C 1998 New 685.69884 218.08155 23.77603
C 1996 Old 1225.74941 303.65572 35.94511
D 1998 New 2299.56170 202.23583 24.34003
D 1996 Old 1852 253.57846 20.52333

```

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
A	08/03/98	7013	Old	0.008	7 / 8	87.500
				0.012	7 / 7	100.000
				0.017	7 / 8	87.500
				0.024	7 / 8	87.500
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/03/98	7014	New	0.008	6 / 8	75.000
				0.012	4 / 8	50.000
				0.017	5 / 8	62.500
				0.024	0 / 8	0.000
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/04/98	7022	Old	0.008	7 / 8	87.500
				0.012	8 / 8	100.000
				0.017	8 / 8	100.000
				0.024	2 / 8	25.000
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/04/98	7023	New	0.008	8 / 8	100.000
				0.012	8 / 8	100.000
				0.017	8 / 8	100.000
				0.024	2 / 8	25.000
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/05/98	7031	Old	0.008	8 / 8	100.000
				0.012	8 / 8	100.000
				0.017	8 / 8	100.000
				0.024	7 / 8	87.500
				0.034	1 / 8	12.500
				0.048	0 / 8	0.000
A	08/05/98	7032	New	0.008	8 / 8	100.000
				0.012	8 / 8	100.000
				0.017	4 / 8	50.000
				0.024	1 / 8	12.500
				0.034	0 / 8	0.000
				0.048	0 / 8	0.000
A	08/06/98	7040	Old	0.008	8 / 8	100.000
				0.012	7 / 8	87.500
				0.017	8 / 8	100.000
				0.024	7 / 8	87.500
				0.034	4 / 8	50.000
				0.048	0 / 8	0.000
A	08/06/98	7041	New	0.008	8 / 8	100.000
				0.012	7 / 8	87.500
				0.017	7 / 8	87.500
				0.024	8 / 8	100.000
				0.034	1 / 8	12.500
				0.048	0 / 8	0.000

Listing of
Raw Data used
for Head-to-
Head
Comparisons
Jennifer R Holdcraft
6/4/99

MREF Task 97-53

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
C	08/04/98	7024	Old	0.005	8 / 8	100.000
				0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	7 / 8	87.500
				0.018	2 / 8	25.000
				0.026	1 / 8	12.500
C	08/04/98	7025	New	0.005	8 / 8	100.000
				0.006	7 / 8	87.500
				0.009	7 / 8	87.500
				0.013	8 / 8	100.000
				0.018	5 / 8	62.500
				0.026	1 / 8	12.500
C	08/05/98	7033	Old	0.005	8 / 8	100.000
				0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	7 / 8	87.500
				0.018	2 / 8	25.000
				0.026	1 / 8	12.500
C	08/05/98	7034	New	0.005	8 / 8	100.000
				0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	7 / 8	87.500
				0.018	5 / 8	62.500
				0.026	2 / 8	25.000
C	08/06/98	7042	Old	0.006	8 / 8	100.000
				0.009	7 / 8	87.500
				0.013	6 / 6	100.000
				0.018	2 / 8	25.000
				0.026	0 / 8	0.000
C	08/06/98	7043	New	0.005	8 / 8	100.000
				0.006	6 / 8	75.000
				0.009	5 / 7	71.429
				0.013	5 / 8	62.500
				0.018	2 / 8	25.000
				0.026	2 / 8	25.000
C	10/14/98	7179	Old	0.005	7 / 8	87.500
				0.006	7 / 8	87.500
				0.009	7 / 8	87.500
				0.013	5 / 8	62.500
				0.018	5 / 8	62.500
				0.026	2 / 8	25.000
				0.037	0 / 8	0.000
				0.052	0 / 8	0.000
C	10/14/98	7180	New	0.005	8 / 8	100.000
				0.006	7 / 8	87.500
				0.009	7 / 8	87.500
				0.013	8 / 8	100.000
				0.018	5 / 8	62.500

MREF Task 97-53

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number Dead / Total	Pct. Dead
C	10/14/98	7180	New	0.026	1 / 8	12.500
				0.037	0 / 8	0.000
				0.052	0 / 8	0.000
D	08/04/98	7026	Old	0.010	8 / 8	100.000
				0.014	8 / 8	100.000
				0.019	7 / 8	87.500
				0.027	7 / 8	87.500
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	08/04/98	7027	New	0.010	8 / 8	100.000
				0.014	7 / 8	87.500
				0.019	8 / 8	100.000
				0.027	8 / 8	100.000
				0.039	8 / 8	100.000
				0.055	0 / 8	0.000
D	08/05/98	7035	Old	0.010	7 / 8	87.500
				0.014	8 / 8	100.000
				0.019	8 / 8	100.000
				0.027	1 / 8	12.500
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	08/05/98	7036	New	0.010	8 / 8	100.000
				0.014	7 / 8	87.500
				0.019	8 / 8	100.000
				0.027	8 / 8	100.000
				0.039	8 / 8	100.000
				0.055	0 / 8	0.000
D	09/01/98	7128	Old	0.010	6 / 8	75.000
				0.014	8 / 8	100.000
				0.019	8 / 8	100.000
				0.027	0 / 8	0.000
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	09/01/98	7129	New	0.028	5 / 8	62.500
				0.039	7 / 8	87.500
				0.055	0 / 8	0.000
				0.078	0 / 8	0.000
				0.110	0 / 8	0.000
				0.160	0 / 8	0.000
D	09/03/98	7131	Old	0.010	6 / 8	75.000
				0.014	7 / 8	87.500
				0.019	7 / 8	87.500
				0.027	7 / 8	87.500
				0.039	0 / 8	0.000
				0.055	0 / 8	0.000
D	09/03/98	7132	New	0.028	7 / 8	87.500
				0.039	8 / 8	100.000

Table A-1. Listing of Experimental Data for Head-to-Head Comparisons

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Number	Pct. Dead
					Dead / Total	
D	09/03/98	7132	New	0.055	6 / 8	75.000
				0.078	0 / 8	0.000
				0.110	0 / 8	0.000
				0.160	0 / 8	0.000

Antitoxin Standard	Experiment Date	Experiment Number	Botulinum Serotype	Number Dead / Total	Pct. Dead	Toxin Dose	Number Survived / Total	Pct. Survived
A	08/26/98	7090	A	0 / 4	0	1000	4 / 4	100
			B	4 / 4	100	50	0 / 4	0
			C	2 / 4	50	50	2 / 4	50
			D	4 / 4	100	50	0 / 4	0
			E	3 / 4	75	50	1 / 4	25
C	08/26/98	7091	A	4 / 4	100	100	0 / 4	0
			B	4 / 4	100	100	0 / 4	0
			C	0 / 4	0	2000	4 / 4	100
			D	4 / 4	100	1000	0 / 4	0
			E	3 / 4	75	100	1 / 4	25
D	09/16/98	7163	A	4 / 4	100	50	0 / 4	0
			B	4 / 4	100	50	0 / 4	0
			C	4 / 4	100	500	0 / 4	0
			D	0 / 4	0	1000	4 / 4	100
			E	4 / 4	100	50	0 / 4	0

from sas dataset mousneut.sas

added in program serospecificity.sas

Listing of Data Used in Serospecificity Analysis

Jennifer R Holdcraft

6/29/99

* Compare circled columns to information in Table 3 of report "Validation of Antitoxin Standards for Serotypes A, C, and D".

StudyNum	StudyDate	Experiment Num	SeroType	LD50	LCBLD50	UCBLD50	LD50ML	Slope	Comment
G1555-53A	7/21/98	7004	A	1.39E-05			144313.7	134.687	Yes (#)
G1555-53A	7/22/98	7010	A	1.11E-05			179757.6	3.56474	Yes (c#)
G1555-53A	8/3/98	7019	A	1.41E-05			141358.4	55.31189	Yes (#)
G1555-53A	8/4/98	7028	A	1.18E-05	3.29E-06	1.58E-05	170142.3	5.178909	Yes
G1555-53A	8/5/98	7037	A					-0.017715	No (b#)
*G1555-53A	8/6/98	7046	A	3.99E-06			501610.2	2.281213	No (b#)
G1555-53A	8/25/98	7084	A	1.29E-05			155434.2	4.429622	Yes (c#)
G1555-53A	8/26/98	7088	A	1.1E-05			182350.4	2.08641	Yes (#)
G1555-53A	8/27/98	7094	A	1.59E-05	7E-06	2.31E-05	125393.1	3.900325	Yes
G1555-53A	9/1/98	7104	A	1.47E-05	9.63E-06	1.86E-05	135731.1	5.672705	Yes
G1555-53A	9/2/98	7115	A	2.06E-05			97143.87	1.58582	Yes (#)
G1555-53A	9/2/98	7123	A	1.78E-05			112049.2	1.873292	Yes (#)
G1555-53A	9/3/98	7126	A	5.68E-06			352305.8	1.699765	No (b#)
G1555-53A	9/10/98	7137	A	7.77E+09			0	-0.02453	Yes (*#)
CG333961A	2/18/99	81002	A	9.68E-06			206604.1	47.4874	No (b#)
CG333961A	4/6/99	81012	A	9.27E-06			215784.1	6.278685	No (b#)
CG333961A	4/8/99	81014	A					0.043854	No (b#)
CG333961A	2/19/99	81004	A	9.38E-06			213189.3	44.38567	No (b#)
CG333961A	2/22/99	81006	A					0	No (b*#)
CG333961A	2/23/99	81008	A	1.7E-05	1E-05	2.46E-05	117751.6	4.181404	Yes
CG333961A	4/5/99	81010	A	1.17E-05			170648	109.302	Yes (#)

* not used
b/c didn't meet acceptable criteria

did not use
notes after
9/12/98

when
Sero A
avidity exp's
performed
7/17-7/22

Listing of Serotype A Potency Data - see following summary for experiments used in calculation of average potency for Serotype A - value to be used in avidity analysis. Exp's not used are noted.

Jennifer R Holdcraft
6/4/99

StudyNum	StudyDate	Priment Num	SeroType	LD50	LCBLD50	UCBLD50	LD50ML	Slope	Comment
G1555-53A	7/21/98	7005	C	0.000238			8394.141	54.91496	Yes (#)
G1555-53A	7/22/98	7011	C	0.000233			8598.823	52.09507	Yes (#)
* G1555-53A	8/3/98	7020	C	7.5E-05			26678.87	3.500156	No (b#)
G1555-53A	8/4/98	7029	C	0.000206	0.000152	0.000261	9701.713	10.02486	Yes
G1555-53A	8/5/98	7038	C	0.000212	0.000169	0.000283	9422.177	9.044164	Yes
G1555-53A	8/6/98	7047	C	0.000272	0.000201	0.000394	7341.047	6.716123	Yes
G1555-53A	8/18/98	7067	C	0.00015	5.72E-05	0.000216	13324.2	3.814954	Yes
G1555-53A	8/25/98	7085	C	0.000377			5308.671	3.994199	Yes (#)
G1555-53A	8/26/98	7089	C	0.000202			9897.187	56.44724	Yes (#)
G1555-53A	8/27/98	7095	C	0.000142			14112.71	47.87823	Yes (#)
G1555-53A	9/1/98	7105	C	0.000209	0.000152	0.000271	9581.798	6.689839	Yes
G1555-53A	9/1/98	7112	C	0.000134			14917.37	2.383539	Yes (#)
G1555-53A	9/2/98	7116	C	0.000333	0.000237	0.008519	6008.071	4.191952	Yes
G1555-53A	9/3/98	7127	C	0.000269	0.000201	0.000352	7433.396	9.111352	Yes
G1555-53A	9/9/98	7144	C	0.000193			10383.51	51.49298	Yes (#)
* G1555-53A	9/9/98	7150	C	0			1.87E+22	0.025612	Yes (#)
G1555-53A	10/14/98	7181	C	0.000247	0.000183	0.000347	8105.298	4.892204	Yes
CG333961A	2/18/99	83002	C	0.000203	0.000103	0.000559	9874.566	3.112475	Yes
CG333961A	4/6/99	83012	C	0.000273	0.000171	0.039239	7326.929	3.051236	Yes
CG333961A	4/8/99	83014	C	0.000131	5.86E-05	0.000177	15247.55	4.924142	Yes
CG333961A	2/19/99	83004	C	0.000147	0.000108	0.000189	13568.54	9.618425	Yes
CG333961A	2/22/99	83006	C	9.77E-05			20476.51	44.70479	No (b#)
CG333961A	2/23/99	83008	C	0.000255	0.000185	0.000435	7834.68	4.796009	Yes
CG333961A	4/5/99	83010	C	0.000142			14112.71	47.87823	Yes (#)

* not used b/c did not pass accept criteria

* not used b/c LD₅₀ = C

did not exp's in dates then 7/9/98 then

no C avidity exp's performed (7141-7143, 7145-7147)

Listing of Serotype C Potency Data - see following summary for experiments used in calculation of average potency for Ser C - value used in avidity analysis. Exp's not used are noted here.

Jennifer R. Holdcraft
6/4/99

StudyNum	StudyDate	Experiment Num	SeroType	LD50	LCBLD50	UCBLD50	LD50ML	Slope	Comment
G1555-53A	7/21/98	7006	D	1.2E-05	7.61E-06	1.62E-05	167171	4.476919	Yes
G1555-53A	7/22/98	7012	D	1.48E-05	1E-05	2.03E-05	135331.1	4.457295	Yes
G1555-53A	8/3/98	7021	D	1.09E-05			183826	53.11804	Yes (#)
G1555-53A	8/4/98	7030	D	9.99E-06	0	1.56E-05	200170.3	2.866323	Yes
G1555-53A	8/5/98	7039	D	1.06E-05			188092.8	57.11785	Yes (#)
G1555-53A	8/6/98	7048	D	1.11E-05			179880.7	54.45054	Yes (#)
G1555-53A	8/19/98	7074	D	1.02E-05	6.54E-06	1.27E-05	196897.8	9.468267	Yes
G1555-53A	8/20/98	7081	D	1.37E-05	6.11E-06	2.14E-05	145735.3	3.596367	Yes
G1555-53A	9/1/98	7130	D	2E-05			99809	2.691411	Yes (c#)
* G1555-53A	9/3/98	7133	D	1.25E-06			1595915	0.704436	No (b#)
G1555-53A	9/15/98	7153	D	1.41E-05	9.2E-06	2.01E-05	142172.5	3.608642	Yes
G1555-53A	9/15/98	7160	D	1.88E-05	1.22E-05	3.29E-05	106336.2	2.892983	Yes
G1555-53A	9/16/98	7162	D	1.39E-05	1.06E-05	1.79E-05	143764.5	7.602791	Yes
G1555-53A	9/17/98	7165	D	1.82E-05	1.37E-05	2.39E-05	110000.5	6.510773	Yes
G1555-53A	9/22/98	7167	D	1.63E-05	1.19E-05	2.17E-05	122341.1	5.187884	Yes
G1555-53A	9/22/98	7174	D	9.86E-06	5.45E-06	1.37E-05	202809.1	3.683719	Yes
G1555-53A	9/23/98	7176	D	1.62E-05	1.12E-05	2.32E-05	123372.7	3.817239	Yes
G1555-53A	9/24/98	7178	D	1.53E-05			130401	4.669925	Yes (c#)
G1555-53A	12/14/98	7186	D	1.77E-05			112710.9	114.7649	Yes (#)
G1555-53A	12/15/98	7191	D	6.96E-06			287171	2.863861	Yes (#)
G1555-53A	12/16/98	7196	D	9.39E-06			213098.2	122.1176	Yes (#)
G1555-53A	1/18/99	7198	D	1.06E-05			187889	0.95955	Yes (c#)
G1555-53A	1/19/99	7200	D	9.39E-06			213098.2	122.1176	Yes (#)
G1555-53A	1/20/99	7202	D	9.8E-06	4.17E-06	1.29E-05	204157.1	5.656712	Yes
G1555-53A	4/19/99	7216	D	0.000202			9913.832	55.50059	Yes (#)
G1555-53A	4/20/99	7218	D	0.000204	0.000159	0.000261	9812.976	7.638789	Yes
G1555-53A	4/22/99	7220	D	0.000148			13477.69	54.17224	Yes (#)
G1555-53A	3/8/99	7211	D	1.11E-05			179521.5	4.321233	Yes (c#)
G1555-53A	3/9/99	7214	D	1.32E-05			151144.4	4.533179	Yes (c#)
G1555-53A	3/4/99	7205	D	1.3E-05	8.48E-06	1.79E-05	154436.1	3.334889	Yes
G1555-53A	3/5/99	7208	D	9.26E-06			215868.2	113.8542	Yes (#)

* not used b/c did not meet the acceptance criteria

did not exp's
in
tes
ter
02/98
when
we
no
D
avidity

exp's were performed 7168-7173)

Listing of Serotype D Potency Data - see following summary for experiments used in calculation of average potency for Ser D - value used in avidity analysis
Exp's not used are noted.

Jennifer R Holdcraft
614199

Calculate Average Potency for Serotypes A,C, and D using All Task 53 Experiments Prior to Avidity Experiments,
Where Reasonable (passed acceptance criteria and LD50ML not 0 or infinity) results were obtained.
Full data for each serotype listed in separate worksheets.

StudyNum	StudyDate	Experiment Nur	SeroType	LD50	LCBLD50	UCBLD50	LD50ML	Slope	Comment
G1555-53A	7/21/98	7004	A	1.39E-05			144313.7256	134.687	Yes (#)
G1555-53A	7/22/98	7010	A	1.11E-05			179757.6416	3.56474	Yes (c#)
G1555-53A	8/3/98	7019	A	1.41E-05			141358.4007	55.31189	Yes (#)
G1555-53A	8/4/98	7028	A	1.18E-05	3.29E-06	1.58E-05	170142.2615	5.178909	Yes
G1555-53A	8/25/98	7084	A	1.29E-05			155434.1983	4.429622	Yes (c#)
G1555-53A	8/26/98	7088	A	1.1E-05			182350.439	2.08641	Yes (#)
G1555-53A	8/27/98	7094	A	1.59E-05	7E-06	2.31E-05	125393.1482	3.900325	Yes
G1555-53A	9/1/98	7104	A	1.47E-05	9.63E-06	1.86E-05	135731.0532	5.672705	Yes
G1555-53A	9/2/98	7115	A	2.06E-05			97143.87413	1.58582	Yes (#)
G1555-53A	9/2/98	7123	A	1.78E-05			112049.1646	1.873292	Yes (#)
							144367.3907	(average)	A

StudyNum	StudyDate	Experiment Nur	SeroType	LD50	LCBLD50	UCBLD50	LD50ML	Slope	Comment
G1555-53A	7/21/98	7005	C	0.000238			8394.141001	54.91496	Yes (#)
G1555-53A	7/22/98	7011	C	0.000233			8598.822635	52.09507	Yes (#)
G1555-53A	8/4/98	7029	C	0.000206	0.000152	0.000261	9701.71267	10.02486	Yes
G1555-53A	8/5/98	7038	C	0.000212	0.000169	0.000283	9422.177035	9.044164	Yes
G1555-53A	8/6/98	7047	C	0.000272	0.000201	0.000394	7341.047395	6.716123	Yes
G1555-53A	8/18/98	7067	C	0.00015	5.72E-05	0.000216	13324.19586	3.814954	Yes
G1555-53A	8/25/98	7085	C	0.000377			5308.670954	3.994199	Yes (#)
G1555-53A	8/26/98	7089	C	0.000202			9897.187297	56.44724	Yes (#)
G1555-53A	8/27/98	7095	C	0.000142			14112.71175	47.87823	Yes (#)
G1555-53A	9/1/98	7105	C	0.000209	0.000152	0.000271	9581.798416	6.689839	Yes
G1555-53A	9/1/98	7112	C	0.000134			14917.36859	2.383539	Yes (#)
G1555-53A	9/2/98	7116	C	0.000333	0.000237	0.000519	6008.070606	4.191952	Yes
G1555-53A	9/3/98	7127	C	0.000269	0.000201	0.000352	7433.396071	9.111352	Yes
G1555-53A	9/9/98	7144	C	0.000193			10383.51487	51.49298	Yes (#)
							9601.772511	(average)	C

StudyNum	StudyDate	Experiment Nur	SeroType	LD50	LCBLD50	UCBLD50	LD50ML	Slope	Comment
G1555-53A	7/21/98	7006	D	1.2E-05	7.61E-06	1.62E-05	167171.0464	4.476919	Yes
G1555-53A	7/22/98	7012	D	1.48E-05	1E-05	2.03E-05	135331.0814	4.457295	Yes
G1555-53A	8/3/98	7021	D	1.09E-05			183825.9513	53.11804	Yes (#)
G1555-53A	8/4/98	7030	D	9.99E-06	0	1.56E-05	200170.2965	2.866323	Yes
G1555-53A	8/5/98	7039	D	1.06E-05			188092.7827	57.11785	Yes (#)
G1555-53A	8/6/98	7048	D	1.11E-05			179880.6786	54.45054	Yes (#)
G1555-53A	8/19/98	7074	D	1.02E-05	6.54E-06	1.27E-05	196897.8206	9.468267	Yes
G1555-53A	8/20/98	7081	D	1.37E-05	6.11E-06	2.14E-05	145735.3274	3.596367	Yes
G1555-53A	9/1/98	7130	D	2E-05			99809.00432	2.691411	Yes (c#)
G1555-53A	9/15/98	7153	D	1.41E-05	9.2E-06	2.01E-05	142172.5378	3.608642	Yes
G1555-53A	9/15/98	7160	D	1.88E-05	1.22E-05	3.29E-05	106336.1999	2.892983	Yes
G1555-53A	9/16/98	7162	D	1.39E-05	1.06E-05	1.79E-05	143764.4799	7.602791	Yes
G1555-53A	9/17/98	7165	D	1.82E-05	1.37E-05	2.39E-05	110000.4909	6.510773	Yes
G1555-53A	9/22/98	7167	D	1.63E-05	1.19E-05	2.17E-05	122341.08	5.187884	Yes
G1555-53A	9/22/98	7174	D	9.86E-06	5.45E-06	1.37E-05	202809.1377	3.683719	Yes
							154955.86102	(average)	D

Listing of Experiments Used in the calculation of Average Potency for Serotypes A, C, and D. Only exp's prior to avidity exp's and exp's that passed acceptance criteria and had LD50's not equal to 0 or ∞ were used.

Jennifer R. Holdcraft

MREF Task 97-53
Table A-3. Listing of Experimental Data for Avidity Analysis

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Toxin Dose (MIP LD50 units/injection)	Number Dead / Total	Pct. Dead
A	09/10/98	7134	New	0.020	721.84	0 / 6	0.0
				0.028	1010.57	0 / 6	0.0
				0.040	1443.67	4 / 6	66.7
				0.057	2057.24	6 / 6	100.0
				0.080	2887.35	6 / 6	100.0
				0.11	3970.10	6 / 6	100.0
* A	09/10/98	7135	New	0.0020	72.18	0 / 6	0.0
				* 0.0028	<u>101.06</u>	0 / 6	0.0
				0.0040	144.37	0 / 6	0.0
				0.0057	205.72	0 / 6	0.0
				0.0080	288.73	6 / 6	100.0
				0.011	397.01	6 / 6	100.0
A	09/10/98	7136	New	0.00021	7.58	0 / 6	0.0
				0.00028	10.11	0 / 6	0.0
				0.00040	14.44	0 / 6	0.0
				0.00057	20.57	0 / 6	0.0
				0.00079	28.51	3 / 6	50.0
				0.0011	39.70	6 / 6	100.0
A	09/10/98	7138	Old	0.010	360.92	0 / 6	0.0
				0.014	505.29	0 / 6	0.0
				0.020	721.84	0 / 6	0.0
				0.028	1010.57	2 / 6	33.3
				0.040	1443.67	6 / 6	100.0
				0.057	2057.24	6 / 6	100.0
A	09/10/98	7139	Old	0.0016	57.75	0 / 6	0.0
				0.0023	83.01	0 / 6	0.0
				0.0032	115.49	0 / 6	0.0
				0.0045	162.41	0 / 6	0.0
				0.0064	230.99	6 / 6	100.0
				0.0091	328.44	5 / 6	83.3
A	09/10/98	7140	Old	0.00013	4.69	0 / 6	0.0
				0.00019	6.86	0 / 6	0.0
				0.00026	9.38	0 / 6	0.0
				0.00038	13.71	0 / 6	0.0
				0.00053	19.13	1 / 6	16.7
				0.00075	27.07	4 / 6	66.7
C	09/09/98	7141	New	0.12	288.05	0 / 6	0.0
				0.17	408.08	0 / 6	0.0
				0.24	576.11	0 / 6	0.0
				0.34	816.15	6 / 6	100.0
				0.49	1176.22	6 / 6	100.0
				0.69	1656.31	6 / 6	100.0
C	09/09/98	7142	New	0.035	84.02	0 / 6	0.0
				0.049	117.62	0 / 6	0.0
				0.069	165.63	0 / 6	0.0
				0.098	235.24	6 / 6	100.0
				0.14	336.06	6 / 6	100.0
				0.20	480.09	5 / 6	83.3

Toxin Dose calculated in program using formula

$$\text{Toxin Dose} = (\text{dose} * \text{avg. potency}) / 4 = (0.0028 * 144367.3907) / 4$$

$= 101.057 \approx 101.06$ ✓
verified by hand Jennifer R. Holdcraft

MREF Task 97-53

Table A-3. Listing of Experimental Data for Avidity Analysis

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Toxin Dose (MIP LD50 units/injection)	Number Dead / Total	Pct. Dead
C	09/09/98	7143	New	0.0053	12.72	0 / 6	0.0
				0.0075	18.00	2 / 6	33.3
				0.010	24.00	3 / 6	50.0
				0.015	36.01	6 / 6	100.0
				0.021	50.41	5 / 6	83.3
				0.029	69.61	6 / 6	100.0
C	09/09/98	7145	Old	0.24	576.11	0 / 6	0.0
				0.34	816.15	0 / 6	0.0
				0.49	1176.22	1 / 6	16.7
				0.69	1656.31	6 / 6	100.0
				0.97	2328.43	6 / 6	100.0
				1.0	2400.44	6 / 6	100.0
C	09/09/98	7146	Old	0.049	117.62	0 / 6	0.0
				0.069	165.63	0 / 6	0.0
				0.097	232.84	0 / 6	0.0
				0.14	336.06	6 / 6	100.0
				0.19	456.08	5 / 6	83.3
				0.27	648.12	6 / 6	100.0
C	09/09/98	7147	Old	0.0069	16.56	0 / 6	0.0
				0.0098	23.52	0 / 6	0.0
				0.014	33.61	3 / 6	50.0
				0.020	48.01	5 / 6	83.3
				0.028	67.21	6 / 6	100.0
				0.039	93.62	6 / 6	100.0
D	09/22/98	7168	New	0.014	542.35	0 / 6	0.0
				0.020	774.78	0 / 6	0.0
				0.028	1084.69	0 / 6	0.0
				0.040	1549.56	0 / 6	0.0
				0.057	2208.12	1 / 6	16.7
				0.080	3099.12	6 / 6	100.0
D	09/22/98	7169	New	0.0011	42.61	0 / 6	0.0
				0.0015	58.11	0 / 6	0.0
				0.0022	85.23	0 / 6	0.0
				0.0031	120.09	0 / 6	0.0
				0.0044	170.45	0 / 6	0.0
				0.0062	240.18	6 / 6	100.0
D	09/22/98	7170	New	0.00022	8.52	0 / 6	0.0
				0.00031	12.01	0 / 6	0.0
				0.00043	16.66	0 / 6	0.0
				0.00062	24.02	5 / 6	83.3
				0.00088	34.09	4 / 6	66.7
				0.0012	46.49	6 / 6	100.0
D	09/22/98	7171	Old	0.014	542.35	0 / 6	0.0
				0.020	774.78	0 / 6	0.0
				0.028	1084.69	1 / 6	16.7
				0.040	1549.56	0 / 6	0.0
				0.057	2208.12	6 / 6	100.0
				0.080	3099.12	5 / 6	83.3

Table A-3. Listing of Experimental Data for Avidity Analysis

Serotype	Experiment Date	Experiment Number	Standard	Dose (u/ml)	Toxin Dose (MIP LD50 units/injection)	Number Dead / Total	Pct. Dead
D	09/22/98	7172	Old	0.0011	42.61	0 / 6	0.0
				0.0015	58.11	0 / 6	0.0
				0.0022	85.23	0 / 6	0.0
				0.0031	120.09	0 / 6	0.0
				0.0044	170.45	0 / 6	0.0
				0.0062	240.18	1 / 6	16.7
D	09/22/98	7173	Old	0.00022	8.52	0 / 6	0.0
				0.00031	12.01	0 / 6	0.0
				0.00043	16.66	3 / 6	50.0
				0.00062	24.02	4 / 6	66.7
				0.00088	34.09	6 / 6	100.0
				0.0012	46.49	5 / 6	83.3

B1-3 Mouse Neutralization Studies

Probit Analyses Of Proportions Of Mice Dead Versus Log10 Antibody Concentration
Old Standard - Common Slope/Common Intercept Model

----- Serotype=A -----

Probit Procedure

Data Set =WORK.D2
Dependent Variable=NDEAD
Dependent Variable=N
Number of Observations= 24
Number of Events = 119 Number of Trials = 191
Observations with Missing Values= 404

Log Likelihood for NORMAL -61.20561023

Goodness-of-Fit Tests

Statistic	Value	DF	Prob>Chi-Sq
Pearson Chi-Square	70.7511	4	0.0000
L.R. Chi-Square	23.9515	4	0.0001

Response Levels: 2 Number of Covariate Values: 6

WARNING: All variances and covariances have been multiplied by the heterogeneity factor H= 17.688. Please check to be sure that the large chi-square ($p < 0.0001$) is not caused by systematic departure from the model. A t value of 2.7764 will be used in computing fiducial limits.

(3 pages)
Probit report output - match these values to those on following pages (for Serotype A only) to verify that values output automatically in a dataset match those in printed output.

Jennifer R Holdcraft
6/28/99

B1-3 Mouse Neutralization Studies

Probit Analyses Of Proportions Of Mice Dead Versus Log10 Antibody Concentration
Old Standard - Common Slope/Common Intercept Model

----- Serotype=A -----

Probit Procedure

Variable	DF	Estimate	Std Err	ChiSquare	Pr>Chi	Label/Value
INTERCPT	1	-9.086576	4.567373	3.957926	0.0467	Intercept
Log10(DOS)	1	-5.7105198	2.801744	4.154268	0.0415	Antibody Concentration

Probit Model in Terms of Tolerance Distribution

MU	SIGMA
-1.5912	0.175115

Estimated Covariance Matrix for Tolerance Parameters

	MU	SIGMA
MU	0.008895	0.001174
SIGMA	0.001174	0.007382

B1-3 Mouse Neutralization Studies

Probit Analyses Of Proportions Of Mice Dead Versus Log10 Antibody Concentration
Old Standard - Common Slope/Common Intercept Model

----- Serotype=A -----

Probit Procedure
Probit Analysis on DOSE

Probability	Log10(DOSE)	95 Percent Fiducial Limits		DOSE 95 Percent Fiducial Limits		
		Lower	Upper			
0.01	-1.18382	.	.	0.06549	0.00000	0.00000
0.02	-1.23156	.	.	0.05867	0.00000	0.00000
0.03	-1.26184	.	.	0.05472	0.00000	0.00000
0.04	-1.28463	.	.	0.05192	0.00000	0.00000
0.05	-1.30316	.	.	0.04976	0.00000	0.00000
0.06	-1.31893	.	.	0.04798	0.00000	0.00000
0.07	-1.33277	.	.	0.04648	0.00000	0.00000
0.08	-1.34515	.	.	0.04517	0.00000	0.00000
0.09	-1.35641	.	.	0.04401	0.00000	0.00000
0.10	-1.36678	.	.	0.04298	0.00000	0.00000
0.15	-1.40970	.	.	0.03893	0.00000	0.00000
0.20	-1.44382	.	.	0.03599	0.00000	0.00000
0.25	-1.47309	.	.	0.03364	0.00000	0.00000
0.30	-1.49937	.	.	0.03167	0.00000	0.00000
0.35	-1.52372	.	.	0.02994	0.00000	0.00000
0.40	-1.54683	.	.	0.02839	0.00000	0.00000
0.45	-1.56919	.	.	0.02697	0.00000	0.00000
ED ₅₀ 0.50	-1.59120	.	.	0.02563	0.00000	0.00000
0.55	-1.61320	.	.	0.02437	0.00000	0.00000
0.60	-1.63556	.	.	0.02314	0.00000	0.00000
0.65	-1.65868	.	.	0.02194	0.00000	0.00000
0.70	-1.68303	.	.	0.02075	0.00000	0.00000
0.75	-1.70931	.	.	0.01953	0.00000	0.00000
0.80	-1.73858	.	.	0.01826	0.00000	0.00000
0.85	-1.77269	.	.	0.01688	0.00000	0.00000
0.90	-1.81562	.	.	0.01529	0.00000	0.00000
0.91	-1.82599	.	.	0.01493	0.00000	0.00000
0.92	-1.83725	.	.	0.01455	0.00000	0.00000
0.93	-1.84963	.	.	0.01414	0.00000	0.00000
0.94	-1.86346	.	.	0.01369	0.00000	0.00000
0.95	-1.87924	.	.	0.01321	0.00000	0.00000
0.96	-1.89777	.	.	0.01265	0.00000	0.00000
0.97	-1.92056	.	.	0.01201	0.00000	0.00000
0.98	-1.95084	.	.	0.01120	0.00000	0.00000
0.99	-1.99858	.	.	0.01003	0.00000	0.00000

B1-3 Mouse Neutralization Studies

Probit Results For Proportions Of Mice Dead Versus Log10 Antibody Concentration
Old Standard - Common Slope/Common Intercept Model

	OBS	Serotype	Standard	Slope	Intercept	Log Likelihood	ED50
*	1	A	Old	-5.71052	-9.08658 ①	-61.2056	0.025633 ✓ checked by hand
	2	C	Old	-4.80261	-8.64341	-70.6443	0.015859
	3	D	Old	-5.51180	-8.92749	-66.2610	0.024005

calculated in
Program

Probit output dataset for 1996 (Old) Standard
- Common Slope / Common Intercept Model

* verify that slope, intercept, and log likelihood in output dataset match probit report output. Only showing 1 case, as values not hand entered but output automatically to dataset - option in SAS probit procedure.

ED50 was calculated in program using formula

$$\log(ED_{50}) = \left(\frac{-\text{intercept}}{\text{slope}} \right) = \frac{-(-9.08658)}{-5.71052}$$

$$\log(ED_{50}) = -1.591200101$$

$$ED_{50} = 10^{-1.591200101}$$

ED50 = 0.025633 ✓ — matches probit output (calculate this value b/c it cannot be output to a dataset automatically in SAS)

Jennifer R Holdcraft
6/28/99

B1-3 Mouse Neutralization Studies

Probit Results For Proportions Of Mice Dead Versus Experiment Day And Log₁₀ Antibody Concentration
 Old Standard - Common Slope/Separate Intercept Model

OBS	Serotype	Standard	Slope	Intercept	Log Likelihood	ED50
1	A	Old	-6.15973	-9.9927	② -56.2355	0.023864
2	A	Old	-6.15973	-10.4120	-56.2355	0.020402
3	A	Old	-6.15973	-9.4946	-56.2355	0.028748
4	A	Old	-6.15973	-9.3436	-56.2355	0.030416
5	C	Old	-4.80193	-8.5808	-70.5125	0.016332
6	C	Old	-4.80193	-8.5808	-70.5125	0.016332
7	C	Old	-4.80193	-8.6719	-70.5125	0.015634
8	C	Old	-4.80193	-8.7181	-70.5125	0.015292
9	D	Old	-5.82202	-8.8964	-62.5781	0.029645
10	D	Old	-5.82202	-9.6003	-62.5781	0.022441
11	D	Old	-5.82202	-9.8568	-62.5781	0.020276
12	D	Old	-5.82202	-9.3845	-62.5781	0.024440

Probit results output to dataset for 1996 (old) Standard for Common Slope / Separate Intercept Model. Compare these results to Table 1 in Validation of Antitoxin Standards report (Old Standard rows only). Data was sorted by date and expnum, so within each serotype 1st value is for first date & expnum, etc.

Jennifer R. Holdcraft

6/28/99 -

Log Likelihood Ratio Test for Differences Between Dates Among Serotypes

Comparison of Common Slope and Intercept-Old Model vs. Common Slope and Separate Intercept-Old Model

OBS	Serotype	Common Slope and Intercept-Old	Common Slope and Separate Intercept-Old	Likelihood Ratio Test	P-Value
* 1	A	① -61.2056	② -56.2355	9.94019	<u>0.00162</u> → 0.002
2	C	-70.6443	-70.5125	0.26365	0.60763
3	D	-66.2610	-62.5781	7.36588	<u>0.00665</u> → 0.007

Log Likelihood Test for Day to Day Variability
(Common Slope/Intercept vs Common Slope / Separate Int.)

in Old Standard. Serotypes A and D significant,
therefore common slope, separate intercept model
adopted for all. P-values given in 1st
paragraph of Results section in "Validation
of Antitoxin Standards.. " report.

Jennifer R Holdcraft

6/28/99

* compare log likelihood values to those on 2
previous pages