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TNO report

TNO-DV2 2005 A217

Diagnosis of exposure to chemical warfare agents
A comprehensive literature survey 1990 – 2005

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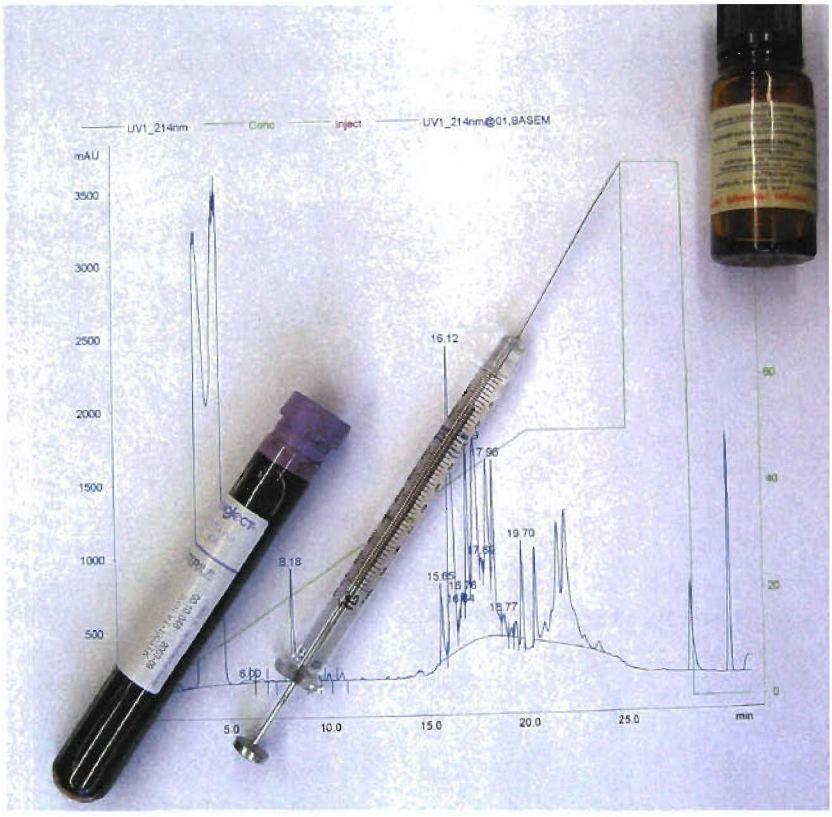
Managementuittreksel

TNO | Kennis voor zaken

Diagnose van blootstelling aan chemische strijdmiddelen



Probleemstelling

Door TNO Defensie en Veiligheid, locatie Rijswijk is de afgelopen jaren een aantal methoden ontwikkeld voor de analyse van adducten van chemische strijdmiddelen in biomedische monsters, waarmee blootstelling aan deze strijdmiddelen kan worden vastgesteld. Deze methoden zijn echter vrij ingewikkeld en alleen geschikt voor gebruik in een goed ingericht laboratorium. Binnen het Nederlandse Ministerie van Defensie bestaat de wens om de ontwikkelde analysemethoden zodanig aan te passen dat deze toepasbaar zijn in een veldlaboratorium. Om dit doel te bereiken is kennis van de "state of the art" op dit gebied van essentieel belang. Dit rapport geeft een overzicht van de momenteel beschikbare methoden voor diagnose van blootstelling aan allerhande chemische strijdmiddelen.

Beschrijving van de werkzaamheden

Er is een literatuurstudie verricht naar de beschikbare methodes voor diagnose van blootstelling aan chemische strijdmiddelen, waarbij tevens is ingeschat of die methodes kunnen worden toegepast in een veldlaboratorium.

Resultaten en conclusies

De literatuurstudie toont aan dat er voldoende methoden zijn waarmee, zonodig na enige modificatie, blootstelling aan chemische wapens kan worden aangetoond. Over het algemeen zullen methoden die gebaseerd zijn op GC-MS makkelijker toepasbaar zijn dan methoden die op LC-MS gebaseerd zijn. De ontwikkelingen op het gebied van miniaturisatie van LC-MS-instrumenten dienen in dit kader goed in de gaten te worden gehouden.
De verkregen resultaten laten tevens zien dat TNO Defensie en Veiligheid, locatie Rijswijk op het gebied van diagnose van blootstelling aan chemische strijdmiddelen een van de meest prominente laboratoria in de wereld is.

Toepasbaarheid

Met het verkregen literatuuroverzicht kan het Ministerie van Defensie zich een goed beeld vormen van de stand van zaken op het gebied van diagnose van blootstelling aan chemische strijdmiddelen. Naast de andere rapporten binnen het pakket "Diagnose" van programma V013, kan dit rapport als advies dienen voor het operationeel stellen van een veldlaboratorium.

PROGRAMMA	PROJECT
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Summary

This report is an update of TNO report PML 2003-A63. In this report an overview is presented of the methods currently available for detection of exposure to a number of chemical warfare agents (CWA), *i.e.*, sulfur mustard, lewisite and nerve agents. Such methods can be applied for various purposes, *e.g.*, diagnosis and dosimetry of exposure of casualties, confirmation of non-exposure, verification of non-adherence to the Chemical Weapons Convention, health surveillance, and forensic purposes.
The methods are either based on mass spectrometric analysis of urine or plasma metabolites that result from hydrolysis and/or glutathione conjugation (Part 1), or on mass spectrometric or immunochemical analysis of CWA adducts with DNA or proteins (Part 2). Several of the methods have been successfully applied to actual cases.

Contents

1 Introduction

[1]Chemical warfare agents (CWA) were used for the first time on a large scale in World War I. The use of the vesicant sulfur mustard, and the pulmonary agents phosgene and chlorine, resulted in 1.3 million casualties (Somani, 1992). Since then, CWA have been used in numerous incidents, *e.g.*, sulfur mustard in the Iran-Iraq conflict and nerve agents against the Kurdish opposition in Iraq (for further reading, see Black *et al.*, 1994), and also in terroristic attacks by the Aum Shinrikyo sect in Japan (Croddy, 1995). After the tragic events of September 11, 2001, the perception of the threat of using CWA during terrorist incidents has increased (Goozner *et al.*, 2002; Rosenbloom *et al.*, 2002). As a result of this continuous threat, CWA have been the subject of a considerable amount of toxicological research, with the ultimate goal of finding defensive measures against these agents (Somani, 1992). In addition, the use or alleged use of CWA in war and terrorism has clearly established an urgent need for biological markers of exposure.

The development of methods for retrospective detection of exposure to CWA is necessary for various reasons. Firstly, such analytical methods can be used to establish firmly whether casualties have indeed been exposed to these chemicals, whereas diagnosis and dosimetry of the exposure will be a starting point for medical treatment of casualties. In this respect, the need for retrospective detection of exposure to CWA has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome" (Noble, 1994). These attempts have led to a general interest in the acute and long-term effects of (low-level) exposure to CWA (Karalliedde *et al.*, 2000; Somani and Romano, 2001). Also, in the case of a terrorist attack with CWA, rapid and reliable diagnosis of the exposure is essential (Noeller, 2001; Murray and Goodfellow, 2002). Conversely, it can be envisaged that confirmation of non-exposure of worried individuals is just as important. Secondly, these methods will be useful for verification of alleged non-adherence to the Chemical Weapons Convention. Especially in this application it appears that maximal retrospectivity, preferably over a period of several months, is essential. Finally, these methods can be used in a variety of other applications, *e.g.*, in unravelling long-term effects of exposure to (low levels of) CW, for health surveillance of workers in destruction facilities of CWA, and for forensic purposes, *e.g.*, for biomonitoring of potential terrorists who are involved in producing or handling CWA.

While methods for sensitive analysis of CWA and their decomposition products in environmental samples have been developed in the last two decades (for a review see Kientz, 1998), methods for biomonitoring of such agents have been available for only a number of years. In this report an overview is presented of methods currently available for biomonitoring of exposure to CWA. Most CW agents are reactive electrophiles. They react with biological nucleophiles, such as water or glutathione, through chemical or enzyme-catalysed reactions to give free metabolites that are excreted in urine. Other metabolic reactions, such as oxidation and S-methylation, also occur. In addition to free

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[1] Parts of the content of this report are reprinted from the following chapters of "Chemical Weapons Convention Chemicals Analysis" (M. Mesilaakso, Ed), 2005, J. Wiley and Sons, with permission from Wiley:

1. Black, R.M. and Noort, D., Methods for the retrospective detection of exposure to toxic scheduled chemicals, Part A: Analysis of free metabolites.
2. Noort, D, and Black, R.M. Methods for the retrospective detection of exposure to toxic scheduled chemicals, Part B: Mass spectrometric and immunochemical analysis of covalent adducts to proteins and DNA.

metabolites, covalent adducts are formed with nucleophilic sites on macromolecules. These may remain in the blood and tissues for much longer, depending on their turnover within the body. Both free metabolites and covalent adducts provide unequivocal biological indicators of exposure to CW agents.
Part 1 of this report will review the metabolism of CW agents and methods for the analysis of free metabolites in biological fluids. In Part 2 covalent adducts formed with proteins and DNA will be reviewed.

2 Part 1. Analysis of free metabolites

2.1 General

2.1.1 Urinary Metabolites

Metabolites excreted in urine offer one of the simplest means of confirming that a casualty has been exposed to a CW agent. Urine has several advantages over other biomedical samples. The major fraction of an absorbed dose of a CW agent is likely to be eliminated as urinary metabolites, a much lesser amount being excreted in the faeces. Urine is a simpler matrix than blood or tissue, and does not require invasive collection. It is generally free from proteins and lipids and is therefore more easily extracted. It is however a very variable matrix in terms of pH (normal range pH 5.5-7) and concentration of inorganic and organic constituents. Conversely, collection of blood and tissue samples (other than hair) is invasive and requires medically competent personnel, but the composition of blood is more consistent than urine. The major disadvantage of urinary metabolites as biological markers is that up to ~90% of the total amount excreted may be eliminated in the first 48-72 h following an exposure.
Figure 1 shows a typical excretion profile for urinary metabolites following systemic administration, a pattern which is likely to be followed after an acute inhalation exposure. It is characterised by a rapid rise to maximum excretion rate, usually within a few hours, with up to 80-90% being excreted within the first 2-3 days. Thereafter a slower elimination phase is usually observed, which may arise from agent repartitioning out of fatty tissues or the slow release of metabolites from covalent adducts of the agent with macromolecules such as protein and DNA. Elimination of metabolites following cutaneous exposure is expected to follow a similar pattern, but the curve may be somewhat flatter resulting from the slower absorption by this route. It is clear from Figure 1 that a much more sensitive analytical technique is required to detect metabolites in urine samples collected several days following an exposure compared to samples collected within the first 48 hours (depending on the dose, self-evidently). This is well illustrated by comparing samples collected from the Iraq-Iran conflict with samples collected from the terrorist attack with sarin in the Tokyo subway.

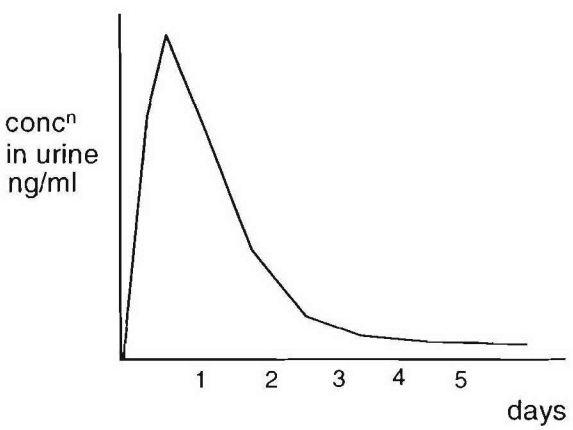


Figure 1 Typical urinary excretion profile of a CW agent metabolite following systemic administration.

2.1.2 *Blood Metabolites*

In addition to urinary metabolites, free metabolites may be detected in blood, provided sampling is undertaken relatively soon after the exposure. Examples are provided in section 3.5.1.3 where blood metabolites were detected in casualties of a terrorist attack in Japan. A disadvantage of blood is that it is a more difficult matrix to work with compared to urine. It is a buffered fluid containing suspended cells, proteins, fats and salts. Fresh blood is easily separated into red cells and plasma (or serum if coagulated), the latter being a much easier matrix. If handled inappropriately (e.g. heating, freezing, agitation, dilution with water), red cells are liable to burst. In most scenarios associated with allegations of CW use, concentrations of free metabolites are likely to be higher in urine. Of much greater importance in blood are adducts with proteins, and these are described in Part 2.

2.1.3 *Analytical Methods*

Most metabolites are small, relatively polar molecules that can be analysed by GC-MS, usually after derivatisation, or underivatised by LC-MS. With most of the MS instruments available, analysis is usually performed using the sensitive selected ion monitoring (SIM) mode. In most cases of allegations of use, analytes are unlikely to be present at concentrations that allow good quality full scan MS data to be obtained, although some modern ion trap and time of flight instruments may have sufficient sensitivity. In the prolonged later phase of excretion of urinary metabolites, single stage MS may not provide sufficiently low limits of detection, and GC or LC combined with tandem MS (MS-MS) is usually required, using multiple reaction monitoring (MRM). MS-MS methods are preferable irrespective of the concentration of analyte as they generally provide a greater degree of specificity (unless full scan EI data can be obtained). Conversion to perfluorinated derivatives and analysis using negative ion chemical ionization mass spectrometry (NICI-MS) has provided the most sensitive methods of analysis for polar metabolites.

Experience has shown that in allegations of CW use, particularly in remote conflicts, samples may be collected several days or even weeks after the event, e.g., after casualties have been moved to hospitals well away from the conflict. In these situations, covalent adducts with biological macromolecules, such as proteins and DNA, offer potentially longer-lived biological indicators. These are discussed in Part 2.

This paper will review the known metabolic pathways of CW agents, excretion profiles where these have been measured, and methods for the analysis of metabolites in urine or blood. Examples are provided of detection in cases of human exposure. The review focuses mainly on sulphur mustard and nerve agents, which represent the greatest global CW threat and for which most analytical methods have been developed.

2.2 Sulphur Mustard

Sulphur mustard remains one of the most important CW agents, despite the fact that it was first used more than 85 years ago. It was used to great effect in WW I, and stockpiled extensively but not used in WW II. More recently it was used in the Iraq-Iran conflict, during which a number of biomedical samples were collected from Iranian casualties. Although not a lethal agent in most circumstances, it causes severe blistering by skin contact and its vapour causes lesions of the eyes and lungs. It can be lethal mostly through secondary infections. The physical properties of sulphur mustard are advantageous for a CW agent and it is one of the easiest agents to produce.

Its mechanism of action with regard to vesicancy is unknown but is assumed to result from its reactions with macromolecules.

2.2.1 *Distribution and Metabolism*

2.2.1.1 *Distribution*

The distribution and rate of elimination of sulphur mustard is partially dependent on the route of exposure and the dose received. Studies with radiolabelled (^{35}S) agent showed that typically from 50-80% of an absorbed dose is eliminated in urine, mostly within the first 3 days (Hambrook et al., 1992, 1993). Hambrook et al. (1992) showed that >70% was excreted in urine following intravenous (i.v.) or intraperitoneal (i.p.) injection in the rat, somewhat less (50-70%) following cutaneous exposure to vapour. The lower percentage following cutaneous exposure resulted from retention in the skin. Urinary excretion of ^{35}S had a half-life of ~1.4 days. Much lower amounts of radioactivity were excreted in faeces, typically 5-15% of the dose, the higher amount after cutaneous application. It was also shown that ^{35}S persisted in blood for more than 6 weeks, associated mainly with the haemoglobin (Hambrook et al, 1993) Radioactivity was rapidly distributed throughout the tissues following i.v. administration in the rat (Maisonneuve et al., 1993).

2.2.1.2 *Metabolism*

Sulphur mustard possesses two electrophilic carbon atoms and its chemistry and metabolism are dominated by their reactions with nucleophiles. In most environments, nucleophilic reactions proceed by an internal SN1 type mechanism, via the episulphonium ion shown in Figure 2. Formation of the episulphonium ion is rate limiting and occurs rapidly in polar solvents (Bartlett and Swain, 1949). Sulphur mustard also possesses an electron-rich sulphur atom that reacts with electrophilic species such as oxidising agents; it participates in formation of the episulphonium ion. In a competitive environment, the episulphonium ion reacts preferentially with 'soft' nucleophiles, such as the thiol function of the cysteine residue in the tripeptide glutathione. Glutathione is relatively abundant in blood and is part of the body's defence mechanism against alkylating agents. Under physiological conditions, reaction with glutathione competes with the most abundant nucleophile water. The metabolism of sulphur mustard is therefore dominated by what are probably chemical (as opposed to enzyme mediated) reactions with glutathione and water, accompanied by metabolic oxidation of the sulphur atom to sulphoxide or sulphone. The various permutations of these reactions (on one or both carbon atoms), combined with divergent metabolic pathways for the reaction products with glutathione, lead to the formation of a large number of metabolites. Mustard also reacts with various sulphydryl, hydroxyl, carboxyl and amino groups on proteins and DNA. These are discussed in Part 2.

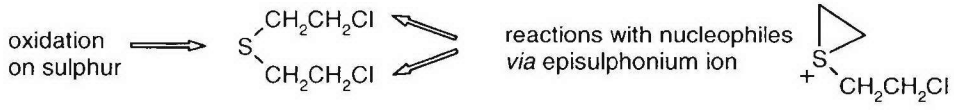


Figure 2 hemical reactivity of sulphur mustard.

Two conflicting metabolism studies were undertaken on sulphur mustard before the advent of modern mass spectrometry. In both studies, ~90% of the dose administered to rats was excreted as urinary metabolites over 5 days, mostly within the first 48 h. Metabolites were tentatively identified by paper chromatography and inverse dilution assay with synthetic compounds. In the first study, in which ^{35}S-mustard was

administered i.v. (Davison et al., 1961) the major metabolites were tentatively identified as the bis-*S*-glutathione conjugate, formed by the reaction of one molecule of sulphur mustard with two molecules of glutathione, plus thiodiglycol (TDG) from simple hydrolysis. The apparent lack of hepatic metabolism in this study was surprising. Some differences were observed in the metabolite profiles obtained from rats and terminal cancer patients. Using a much lower dose, a greater proportion of radioactivity was retained in cancer patients. In a second study using i.p. administration, hepatic metabolism was much more evident, the major metabolite being tentatively identified as the bis-*S*-(*N*-acetylcysteine) (or bis-mercapturic acid) conjugate of mustard sulphone, formed by the expected metabolism of the bis-*S*-glutathione conjugate (Roberts and Warwick, 1963). Also detected was a very polar metabolite [probably thiodiglycol sulphoxide (TDGO)- see below], which was also formed after administration of TDG.

Unequivocal identification of the bis-(*N*-acetylcysteine) conjugate (**1**) (Figure 3) was later made by Black et al. (1992), also using i.p. administration in the rat. Repetitive liquid chromatography was used to isolate metabolites and identification was by tandem mass spectrometry, using desorption chemical ionisation, and comparison with synthetic samples. Also identified at lower concentration, was the analogous conjugate of mustard sulphoxide, plus three mono-conjugates derived from the reaction of mustard with one molecule of glutathione. The metabolites identified from the glutathione pathways are shown in Figure 3. The most interesting metabolites identified were (**2**) and (**3**), derived from further metabolism of the bis-glutathione conjugate by the β-lyase pathway, which proceeds through cleavage of the S-C bond in the cysteine moiety followed by S-methylation (Bakke and Gustaffson, 1984). As with the earlier studies, a large number of metabolites were evident from LC radiochromatograms, and undoubtedly a number of additional metabolites could be identified using modern LC-MS techniques. In particular, no mono-N-acetylcysteine conjugates derived from hemi-mustard (i.e. containing a CH_2CH_2OH moiety) were isolated but these are expected to be significant metabolites.

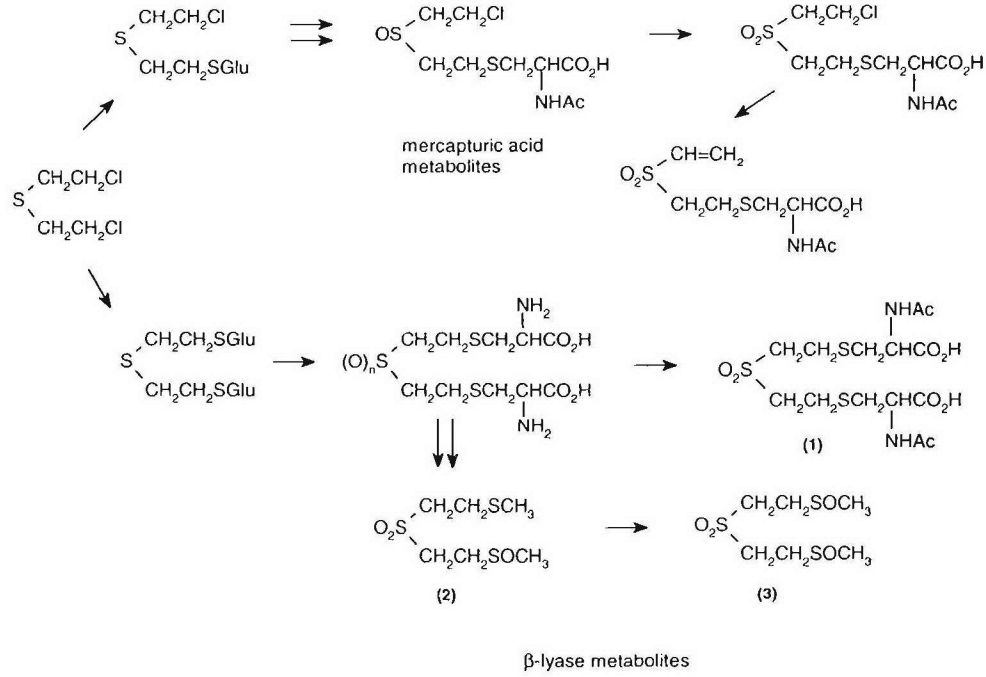


Figure 3 Metabolites of sulphur mustard identified by mass spectrometry, derived from an initial reaction with glutathione and metabolised by divergent mercapturic acid and β-lyase pathways.

Metabolites derived from hydrolysis in the rat were TDG (**4**) and its sulphoxide TDGO (**5**), the former being a minor metabolite and the sulphoxide a major one. Also identified as a very minor metabolite was the much less reactive mustard sulphoxide. These are shown in Figure 4.

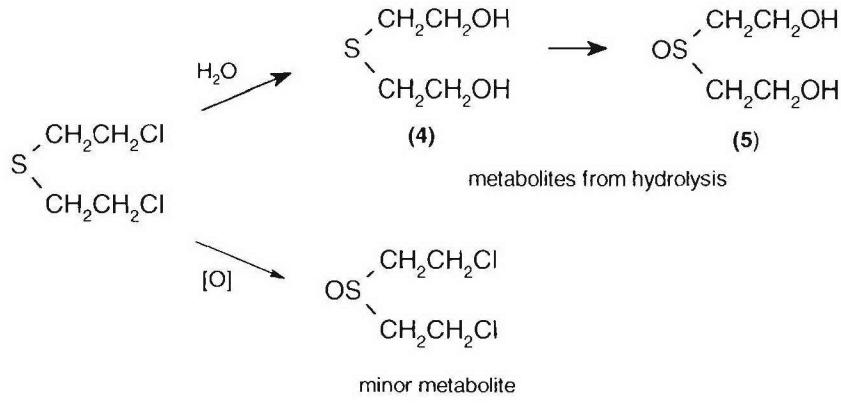


Figure 4 Metabolites of sulphur mustard identified by mass spectrometry, derived from hydrolysis and oxidation (**4** = thiodiglycol; **5** = thiodiglycol sulfoxide).

Urinary excretion profiles were measured for TDG, TDGO and β-lyase metabolites in the rat after percutaneous administration (Black et al., 1992). Levels of TDG increased up to ten fold by treatment of the urine with hydrochloric acid, indicating the presence of acid labile conjugates; TDGO levels rose by only a small amount (~30%) after acid treatment. Levels of TDGO were consistently several times higher than those of TDG. Free TDG, TDG liberated by acid treatment, and TDGO excreted over 8 days accounted for <0.3%, 1-1.5% and 3.4-4.3% of the applied dose respectively. In a separate series of experiments, the hydrolysis products were detected as the single analyte TDG and the β-lyase metabolites as a single reduced analyte (see below). Excretion of hydrolysis products over 8 days accounted for 3.7-13.6% of the applied dose and β-lyase metabolites 2.5-5.3% (there was considerable variation between animals). The excretion of β-lyase metabolites showed a much sharper decline than hydrolysis products (Figure 5), suggesting that the prolonged excretion of hydrolysis products results from TDG being slowly liberated from adducts with macromolecules (see Part 2).

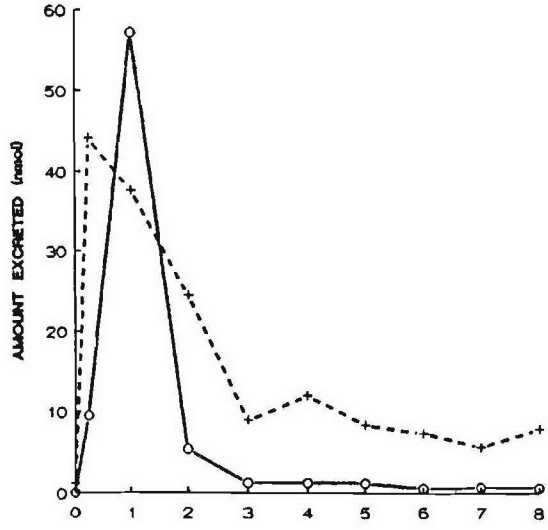
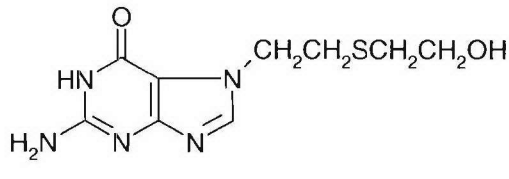


Figure 5 Urinary excretion profiles of metabolites derived from hydrolysis and the β-lyase pathway in the rat following cutaneous application of sulphur mustard (dose = 2 μmol/animal, data points mean of 4 animals; dotted line = hydrolysis products, solid line = β-lyase metabolites).

One aspect of the metabolism of sulphur mustard that appears to be unresolved is the relative importance of TDG and TDGO as excretion products in man. In metabolism studies in rats, TDGO was present in much higher concentrations than TDG. Furthermore, when ^{35}S $^{13}C_4$-TDG was administered (i.p.) to rats, approximately 90% of the administered dose was excreted in the 0-24 h urine, mostly (≥90%) as TDGO (Black et al., 1993). Thiodiglycol sulphone, *S*-(2-hydroxyethylthio)acetic acid and *S*-(2-hydroxyethylsulphinyl)acetic acid were identified as minor metabolites resulting from oxidation on sulphur or carbon. Only 0.5-1% of the TDG was excreted unchanged. Black et al. (1995) found the sulphoxide to be the prevalent form in cases of human exposure, but Jakubowski et al. (2000) detected quite high concentrations of TDG following an accidental laboratory exposure to sulphur mustard. As will be discussed below, TDG and TDGO are less than ideal as biological indicators of poisoning because true blanks in human urine cannot be obtained.

Two additional urinary excretion products have been reported for sulphur mustard, both presumably derived from an initial reaction with a macromolecule. N7-(2-Hydroxyethylthioethyl)guanine (**6**) was identified in the urine of guinea pigs treated with sulphur mustard (1 mg/kg, i.v.). This metabolite is derived from the breakdown of alkylated DNA (see Part 2) (Fidder et al, 1996). Excretion was maximal during the first 2-3 h (50 ng/ml) decreasing to 10 ng/ml in the interval 34-48 h. The imidazole derivative (**7**) was identified in plasma and urine following percutaneous administration of sulphur mustard in the pig (Sandelowsky et al. 1992). This metabolite was presumed to be derived from an initial reaction with a protein histidine residue.



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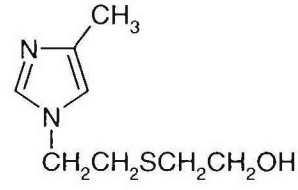


(7)

2.2.1.3 Non-Metabolised Sulphur Mustard

Although an absorbed dose is expected to be fully metabolised, sulphur mustard is a highly lipophilic molecule that will partition into fatty tissues. Surprisingly high concentrations of sulphur mustard were reported in various organs and tissues, particularly abdominal fat, removed post mortem from a lethal casualty of sulphur mustard poisoning (Drasch et al., 1987). This subject had absorbed a large dose of agent. A biomedical sample that should not be overlooked is hair. A United Nations investigation (Anonymous, 1986) reported the detection of unchanged sulphur mustard in hair from an alleged casualty of mustard poisoning. In the laboratory of one of the authors, some evidence was found to suggest that residues of sulphur mustard may become trapped within the hair matrix. A sample of human hair was exposed to sulphur mustard vapour and thoroughly extracted with solvent. It was then digested with the enzyme Pronase in the presence of dithiothreitol (which breaks disulphide linkages) in an effort to detect covalently bound residues. Surprisingly, mustard adducts with the dithiothreitol were observed.

2.2.2 Analytical Methods for Sulphur Mustard and its Metabolites

Analytical methods have been reported for unchanged agent and six of the urinary excretion products described above. These are: TDG, TDGO, the bis N-acetylcysteine conjugate (**1**), two β-lyase metabolites (**2**) and (**3**), and the guanine adduct (**6**). These methods have been applied to animal and/or human exposures to sulphur mustard.

2.2.2.1 Unmetabolised Agent

Trace analysis of sulphur mustard is relatively simple, although it is unlikely to be present in biomedical samples collected in most scenarios. The agent can be extracted from samples, e.g. homogenised tissues (Drasch et al., 1986) or blood (Maisonneuve et al., 1992) with organic solvents such as dichloromethane or ethyl acetate. Simple clean up can be afforded using an SPE cartridge. Analysis can be performed by single stage GC-MS (with SIM) or by GC-MS-MS (with MRM), using a non-polar capillary column.

2.2.2.2 Thiodiglycol

TDG, and particularly the more polar TDGO, are challenging metabolites to analyse in complex aqueous matrices. TDG can be analysed by GC-MS or LC-MS without derivatisation, but these methods generally lack sensitivity. For limits of detection in the low parts per billion (ppb) range, thiodiglycol must be isolated from urine or blood in a non-aqueous solvent, derivatised, and analysed by GC-MS or GC-MS-MS. Derivatives commonly used for environmental analysis are the trimethylsilyl (TMS) and *tert*-butyldimethylsilyl (TBDMS) ethers (Black and Muir, 2003) These may be applicable to the analysis of biomedical samples collected soon after a relatively high exposure to sulphur mustard, but they are not well suited to analysis at low ppb levels. For biomedical sample analysis, pentafluorobenzoyl and heptafluorobutyryl esters have been used. Black and Read (Black and Read, 1988, 1995) analysed TDG as its bis-pentafluorobenzoyl derivative using NICI-MS or NICI-MS-MS for detection. Urine, blood or plasma, with $^{2}H_{4}$-TDG added as internal standard, was loaded onto a solid absorbent tube (which provides a large surface area for liquid-liquid extraction) and extracted with ethyl acetate. The extracts were cleaned up on Florisil (urine) or C18 (blood, plasma). After concentration, the dried residues were derivatised with pentafluorobenzoyl chloride (pyridine, ambient temperature, 5 min). The NICI mass spectrum of TDG-bis-pentafluorobenzoate shows only one significant ion, the molecular ion at *m/z* 510. With the ion current concentrated in this single high mass ion

(plus isotope peaks), the method is very sensitive; detection limits were ~1 ng/ml. A disadvantage is that it is limited for confirmatory analysis, and a second analysis on a different GC column would be recommended in cases of allegations of CW use (and detection of a second biological indicator). A small increase in selectivity can be achieved using GC-MS-MS (Black and Read, 1995). Collision induced dissociation (CID) of *m/z* 510 gave only non-specific product ions $[C_6F_5CO_2]^-$, *m/z* 211 and $[C_6F_5]^-$, *m/z* 167, derived from the pentafluorobenzoyl moiety. However, significantly cleaner chromatograms were obtained by monitoring the parent ion of *m/z* 167, compared to single stage MS using SIM. For standards, 0.2 pg injected could easily be detected, indicating a theoretical limit of detection of ~0.1 ng/ml, making it the most sensitive method to have been reported for TDG. The true detection limit in urine could not be determined because of very low background levels (usually < 1 ng/ml) of TDG. A second disadvantage of the method is that pentafluorobenzoyl chloride does not give a clean reaction product, and the procedure is only suitable for use with NICI-MS. A recent modification, for analysing TDG released by hydrolysis from macromolecules, includes a partial clean up on C18 after derivatisation (Korte et al., 2002; Capacio et al., 2004).

Jakubowski et al., (1990) analysed TDG in urine as its bis-heptafluorobutyryl (HFB) derivative. $^2H_8$-TDG was added as internal standard and the urine incubated for 1 h with glucuronidase with sulphatase activity to hydrolyse any conjugates present. The urine was then adjusted to pH 3-4, concentrated to dryness, reconstituted in ethyl acetate, and derivatised with heptafluorobutyric anhydride (60°C, 1 hour). The HFB derivative produced analytically useful fragment ions at *m/z* 300 and 301 (70 and 50 % relative abundance) resulting from loss of the $C_3F_7CO_2$ moiety. Using SIM, TDG could be detected in urine down to concentrations of 1 ng/ml. The advantage of this method is that it is usable on basic benchtop GC-MS systems. A disadvantage is the need to concentrate urine to dryness, which is time consuming and a potential source of error through evaporative losses and residual traces of water interfering with the derivatisation. The HFB derivative also shows promise under positive CI conditions (Black et al., 2001).

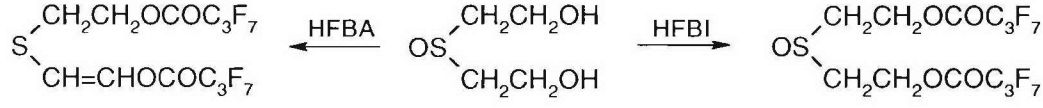
Wils et al. (1985, 1988) previously reported an entirely different approach to TDG analysis. TDG in urine was converted back to sulphur mustard by treatment with concentrated HCl. The sample treatment is less straightforward than the methods described above, but analysis as sulphur mustard is facile. Urine, plus $^2H_8$-TDG as internal standard, was cleaned up by elution through two C18 cartridges. Concentrated HCl was added and the sample stirred and heated at 120°C. Nitrogen was blown over the solution and sulphur mustard isolated from the headspace by adsorption onto Tenax-TA. The method was used to detect TDG in urine from casualties of CW attacks (see below). A disadvantage of this method is that it may convert metabolites other than TDG to sulphur mustard. This is supported by the detection of relatively high levels of analyte in urine from control subjects. Vycudilik (1987) used a similar procedure, but recovered the mustard by steam distillation and extraction.

2.2.2.3 *Thiodiglycol sulphoxide*

Trace analysis of TDGO is more difficult than TDG due to the high polarity of the sulphoxide moiety. TDGO is considerably more polar than TDG and the corresponding sulphone, making selective isolation from aqueous matrices difficult. Clean-up is problematic because it is highly retained on normal phase silica, requiring very polar solvents for elution, and is not retained under reversed phase conditions. TDGO can be

analysed by LC-MS but, as with TDG, detection limits are modest. Derivatisation of TDGO for GC-MS analysis can be problematic because the sulphoxide oxygen is an additional nucleophilic site for reaction.

Black and Read (1991) developed a procedure that extracted TDGO with ethyl acetate - methanol (100:7) after absorption of urine, with $^{2}H_4$-TDGO added as internal standard, onto a solid absorbent. The extract was eluted through a Florisil cartridge, concentrated to dryness, and derivatised with pentafluorobenzoyl chloride as for TDG. This procedure is not ideal because the high polarity of the solvent required for extraction produces an extract containing high levels of extraneous materials. A second disadvantage is that the derivative formed is the same as that produced from TDG, i.e., the sulphoxide function is reduced. This makes it difficult to distinguish the sulphoxide from TDG at trace levels other than by selective extraction. The detection limit is similar to that for TDG, but more extraneous peaks are observed in the chromatogram due to the solvent required for extraction.
Derivatisation of TDGO is complicated because three major types of derivative are formed, depending on the reagent and conditions (Black et al., 2001). These result from selective derivatisation of the hydroxyl groups with preservation of the sulphoxide function, reduction to the corresponding thiodiglycol derivative, and Pummerer-type rearrangement to derivatives or elimination products of 1-hydroxythiodiglycol. For example, the reaction with heptafluorobutyric anhydride gave a derivative tentatively identified as (**8**), derived from a Pummerer-type rearrangement and elimination (it is not yet clear if the elimination occurs during the derivatisation or in the hot injector).
A similar product was obtained with trifluoroacetic anhydride.
Heptafluorobutyrylimidazole (HFBI) gave the sulphoxide derivative (**9**) as the major product. Reduction to the TDG derivative was observed with heptafluorobutyryl chloride and flophemsyl chloride. These derivatisation reactions are currently being evaluated for biomedical sample analysis.

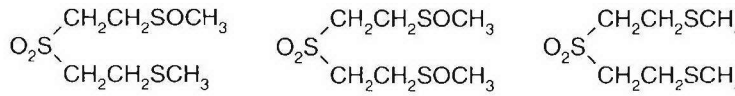


(8) **(9)**

An alternative approach to TDGO analysis is to reduce it to TDG by treating urine with titanium trichloride (Black and Read, 1991). This provides a convenient procedure for the combined analysis of hydrolysis products and β-lyase metabolites (see below).

2.2.2.4 β-Lyase metabolites

The two metabolites identified in the rat derived from the β-lyase pathway, (**2**) and (**3**), have one and two sulphoxide groups respectively. These promote elimination reactions on hot surfaces in the GC-MS, as well as imparting higher polarity to the molecules. Reduction of the sulphoxide groups to sulphide with titanium trichloride (Black et al., 1991) produces a single analyte (**10**), which is efficiently extracted from urine on a C8 SPE cartridge, and gives a sharp GC peak. The hexadeuterated bis-SOC[^{2}H]$_3$ analogue of (**3**) was added to urine as internal standard.



Single stage GC-MS analysis, using positive ammonia CI and SIM of $[M+NH_4]^+$, *m/z* 232, gave a detection limit of 2 ng/ml (Black et al., 1991). GC-MS-MS provided a much lower detection limit (0.1 ng/ml), monitoring the fragmentation *m/z* 232 → $[MeSCH_2CH_2]^+$, *m/z* 75, resulting from a substantial reduction in chemical background (Black and Read, 1995). The chromatograms obtained from single stage and tandem MS are compared in Figure 6. This improved limit of detection allowed the detection of β-lyase metabolites in the urine of seven CW casualties (see below). The method was originally developed for use on a triple sector quadrupole mass spectrometer, and a detection limit of 0.1 ng/ml was easily achievable. However, transfer of the method to a benchtop GC-ion trap MS system has resulted in higher and rather inconsistent limits of detection (0.5-1 ng/ml). A possible reason is that the analyte, which contains three sulphur atoms, is sensitive to hot surfaces. Recently, Boyer et al. (2004) reported a detection limit for **10** of 0.25 ng/ml by using GC tandem MS; the methodology could even be improved by Young et al (2004) to 0.038 ng/ml urine.

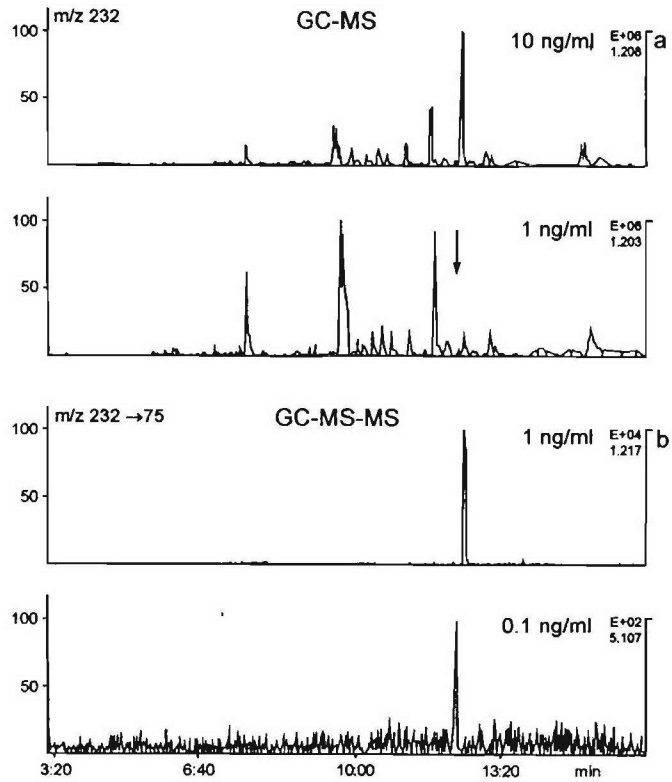


Figure 6 GC-MS-SIM (upper) and GC-MS-MS-MRM (lower) chromatograms for reduced β-lyase metabolites, showing a >50 fold improvement in signal-to-noise ratio using GC-MS-MS.

The use of titanium trichloride for the reduction of sulphoxide groups allows a combined approach to the analysis of urine for metabolites derived from hydrolysis and the β-lyase pathway, as shown in Figure 7 (Black et al., 1992; Black and Read, 1995).

Urine is treated with titanium trichloride, divided into two aliquots, and analysed for TDG and the reduced β-lyase product (**10**) as described above.

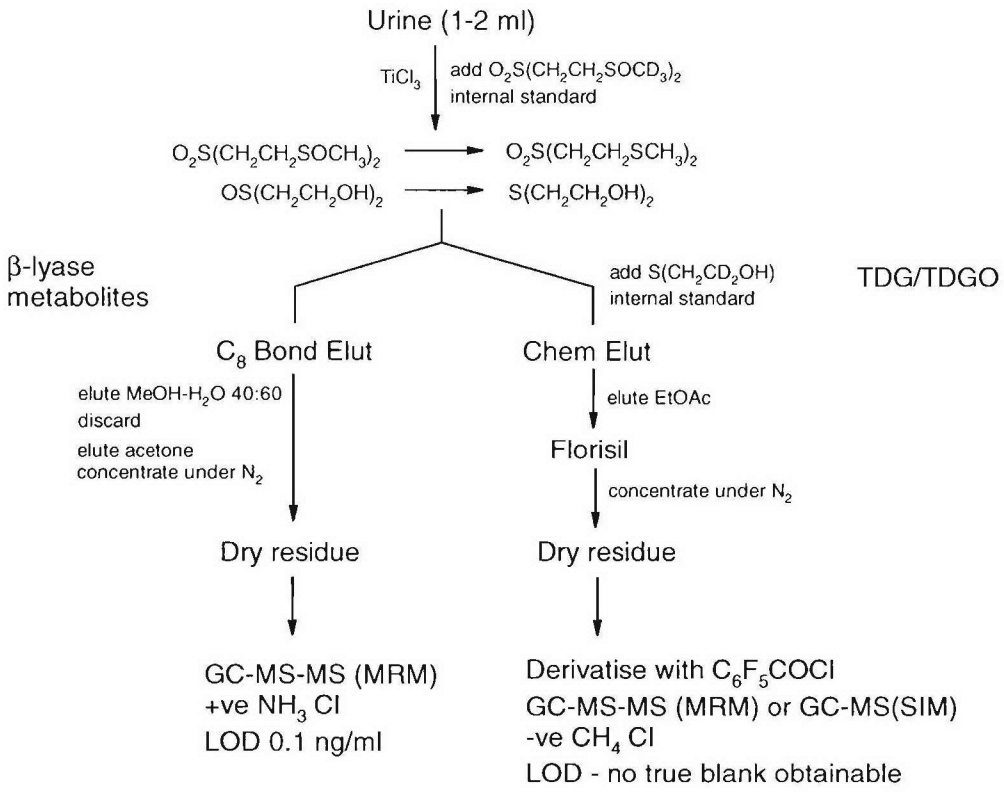
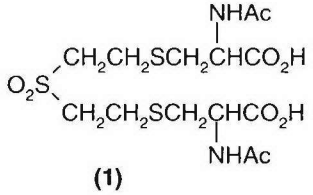


Figure 7 Scheme for the combined analysis of urine for sulphur mustard metabolites derived from hydrolysis and the β-lyase pathway.

An alternative to GC-MS-MS of the reduced product (**10**) is direct LC-ESI-MS-MS of (**2**) and (**3**). The analytes can be isolated from urine by SPE on a hydroxylated polystyrene-divinylbenzene (PS-DVB) polymeric cartridge. Using a sensitive triple sector quadrupole LC-MS-MS system, detection limits of 0.1 ng/ml have been achieved using positive ESI and MRM (Read and Black, 2004).

2.2.2.5 *Bis-(N-Acetylcysteine) Conjugate of Mustard Sulphone*



(1)

Attempts to develop a GC-MS method for this metabolite were unsuccessful, no doubt because of thermal instability. An LC-MS-MS method using thermospray ionisation, after derivatisation to the dimethyl ester, gave a modest detection limit of 25 ng/ml, again probably due to poor thermal stability, in this case in the thermospray ion source (Black and Read, 1995). A substantial improvement has recently been achieved (detection limit 1 ng/ml) using LC-ESI-MS-MS without derivatisation (Read and Black,

2004b). Concentration from acidified urine was achieved on a polymeric SPE cartridge and detection was by negative ion ESI using MRM.

2.2.2.6 N7-(2-Hydroxyethylthioethyl)guanine

The guanine adduct (**6**) can be isolated from urine by SPE on C18. GC-MS analysis of the adduct was problematic. Derivatisation with heptafluorobutyric anhydride and pentafluorobenzoyl bromide were unsuccessful and the TBDMS derivative had poor GC properties. A sensitive method was developed for the underivatised compound using LC-ESI-MS-MS, monitoring the fragmentation MH^+, *m/z* 256 → $[CH_2CH_2SCH_2CH_2OH]^+$, *m/z* 105. The detection limit was 8 pg injected (S/N 5:1), 0.2 ng/ml in urine (Fidder et al, 1996).

2.2.3 Application to Human Casualties

Most of the analytical methods reported above have been successfully employed in cases of human poisoning, either casualties of the Iraq-Iran and Iraq-Kurd conflicts, or cases of accidental poisoning.

2.2.3.1 Unmetabolised Agent

As part of a UN investigation into the use of chemical weapons in the Iraq-Iran war, sulphur mustard was detected in a dichloromethane extract of the hair of one casualty, but not in another. The sample was believed to have been collected soon after the exposure. Identification was by full scan GC-MS, the concentration estimated as 0.5-1 ug/g (ppm). Drasch et al (1987) reported surprisingly high concentrations of sulphur mustard in tissues removed post mortem from a CW casualty who died 7 days after exposure. Qualitative analysis was performed using full scan GC-MS and quantitative analysis by electrothermal atomic absorption spectrometry of a mustard-gold complex. Concentrations of mustard reported in various organs (lung. kidney, brain etc.) ranged from 0.8 - 10.7 µg/g, and in fatty tissues including skin up to 15.1 µg/g. Concentrations of 1.1 µg/ml were reported in blood but none was detected in urine or blister fluid. These results suggest that mustard sequestered in fatty tissues may remain in the body longer than previously suspected from animal studies, though the concentrations in blood are surprising.

2.2.3.2 TDG and TDGO

Wils et al. (1985, 1988) reported the detection of TDG in urine from Iranian casualties, by conversion of TDG back to sulphur mustard with HCl. The alleged attacks occurred in 1984 and 1986, and samples were collected after casualties had been removed to hospitals in Utrecht and Ghent. Urine was immediately frozen on collection for transport to the analytical laboratory and then stored at -23°C. Concentrations of TDG detected in urine collected 5-10 days after the alleged attack were mostly in the range 5-100 ng/ml, but in one casualty 330 ng/ml (this patient died one day after admission). A second set of samples collected from one of the groups 18 days after the reported exposure showed very low levels of analyte (in the range 3-8 ng/ml).
A complicating factor was the presence of low levels of analyte in control urine samples. In most cases these were in the range 1-12 ng/ml. However, a concentration of 55 ng/ml was detected in one sample from a control patient in the first investigation, and 21 ng/ml was detected in a control sample in the second. These values were closer to those detected for TDGO in normal urine (Black and Read, 1991) and suggest that the method also converts other urinary excretion products to sulphur mustard. When TDG was analysed directly as its bis-pentafluorobenzoyl or bis-heptafluorobutyryl derivative, concentrations found in control samples were generally <1 ng/ml, although

no true blank could be obtained (Jakubowski et al., 1990; Black and Read, 1988; Black and Read, 1995).
TDG and TDGO (after reduction to TDG) were detected retrospectively in urine samples taken from three of the same casualties, collected 8 days after the exposure and after storage at -20°C for 5 years. Concentrations were in the range 27-72 ng/ml, compared to a concentration of 11 ng/ml in a control sample. In two casualties accidentally exposed to sulphur mustard from a WW I munition, concentrations in urine collected 2-3 days after the exposure were TDG 2 ng/ml (in both), TDGO 69 ng/ml and 54 ng/ml (mean of 3 determinations) (Black and Read, 1995).
These are shown in Table 1.

Jakubowski et al. (2000) were able to monitor the excretion of TDG in a subject accidentally exposed to sulphur mustard in a laboratory. The casualty developed blisters on hands and arms (<1% of body area) and erythema on his face and neck (<5% of body area). Urine was collected over a 10 day period. A maximum excretion rate of TDG of 20 ug per day was observed between days 3 and 4, the highest concentration being ~65 ng/ml. It was noted that the total amount of urine produced for analysis during the first 3 days was low. Concentrations >10 ng/ml were detected in urine for 7 days after the exposure. The half-life of excretion was estimated as 1.18 days. The total amount of TDG excreted over the 10 day period was 243 ug. There was mass spectrometric evidence of oligomers of TDG [e.g. $(HOCH_2CH_2SCH_2CH_2)_2O$] in a sample of blister fluid. A crude estimate of the cutaneous exposure was ≥49 mg.

Neither TDG nor TDGO are ideal biological markers of exposure because of the very low concentrations present in the urine and blood of normal (unexposed) subjects. TDG appears to be present in urine usually at levels <1 ng/ml, but higher concentrations (up to 16 ng/ml) have been detected in blood (Black and Read, 1988). TDGO was detected at concentrations mainly in the range 1-10 ng/ml in normal human urine, but in one subject was as high as 36 ng/ml. (Black and Read, 1991). The source of these background levels is unknown but may be dietary.

Table 1 Concentrations of metabolites found in the urine of two subjects accidentally exposed to sulphur mustard from a WW 1 munition; urine samples were taken between 2-3 days.

Metabolite	Amount detected (ng/ml)		
	Subject 1	Subject 2	Control
TDG (free & conjugated)	2,2	2,2	<1
TDGO	70, 66, 70	42, 42, 50	5, 5
β-lyase metabolites	43, 42, 42	56, 57, 55	<0.1
mustard sulphone bis(N-acetylcysteine) conjugate*	~1	~1	<1

* analyses performed after a further 10 years storage at ≤15°C

2.2.3.3 *β-Lyase metabolites*

β-Lyase metabolites have been detected in urine samples from nine subjects. Five were from Iranian casualties (three of which were also analysed for TDG and TDGO - see above), two from Kurdish casualties, and two from the subjects accidentally exposed from a WW I munition. The urine from one of the Iranian casualties contained

exceptionally high levels of β-lyase metabolites (~220 ng/ml). This subject died one day after admission to hospital, and is believed to be the casualty reported by Wils et al. (1988) to be excreting very high levels of TDG. Much lower levels (0.5-5 ng/ml) were found in urine from the other four Iranian casualties. Samples from two Kurdish casualties of mustard poisoning, collected 13 days after the reported attack, contained concentrations of β-lyase metabolites close to the limit of detection (0.1-0.3 ng/ml). GC-MS-MS MRM chromatograms are shown in Figure 8. These analyses clearly demonstrated the advantages of sub-ng/ml detection limits as these low concentrations would not have been detected using single stage GC-MS. The analyses also demonstrated the advantages of β-lyase metabolites over hydrolysis products as unequivocal indicators of mustard poisoning. True blanks have always been obtained for β-lyase metabolites in control urine samples. The larger quantity of urine obtained from the two subjects accidentally exposed to sulphur mustard from a WW1 munition allowed a comparison of concentrations of β-lyase metabolites and hydrolysis products. The results are shown in Table 1. In both subjects, substantial amounts of both types of metabolite were excreted but true control blanks were only obtained with β-lyase metabolites. Very recently, the β-lyase metabolites in these samples were determined by LC-ES-MS-MS; in both cases, the monosulphoxide (**2**) appeared to be present in greater concentration than the disulphoxide (**3**) (Read and Black, 2004).

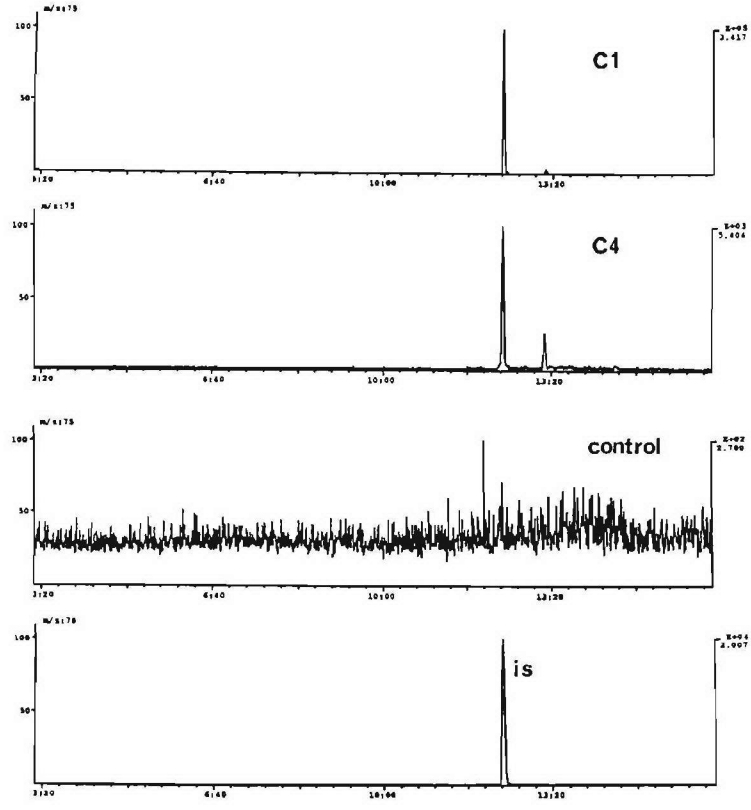


Figure 8 GC-MS-MS-MRM chromatograms showing the detection of β-lyase metabolites in Iranian and Kurdish casualties.

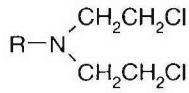
2.2.3.4 *Bis-(N-Acetylcysteine) Conjugate of Mustard Sulphone*

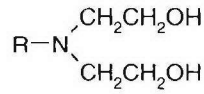
An initial analysis of urine from the two accidental casualties reported above, using LC-thermospray-MS-MS, was negative for the bis-(N-acetylcysteine) conjugate (**1**).

Very recently, these samples have been re-analysed using the LC-ES-MS-MS method reported in section 2.2.3.2. Low concentrations (~1 ng/ml) were detected in both samples (Read and Black, 2004b). These are lower than would have been expected from animal studies but the samples had been stored frozen for 12 years and thawed and refrozen on several occasions.

2.3 Nitrogen Mustards

Three nitrogen mustards (**11a-c**) are included in Schedule 1 of the CWC. Although they are potent vesicants and simple to produce, nitrogen mustards are generally regarded as less effective than sulphur mustard as CW agents. They have accordingly attracted much less attention and few analytical methods have been reported for their analysis. HN-2 has been used as an anticancer agent and some metabolism studies have been reported in this context.





(11a) HN-1, R = Et
(11b) HN-2, R = Me
(11c) HN-3, R = CH_2CH_2Cl

(12a) R = Et
(12b) R = Me
(12c) R = CH_2CH_2OH

2.3.1 Metabolism

Nitrogen mustards are hydrolysed to the corresponding ethanolamines (12a-c) in aqueous media and, like TDG, these are expected to be excretion products following exposure. No detailed mass spectrometric studies of the in vivo metabolism of nitrogen mustards have been reported. Lemire et al. (2003) referred to unpublished studies that showed substantial amounts of free (unconjugated) N-ethyl and N-methyldiethanolamine were present in 24 h and 48 h urine from rats exposed to HN-1 and HN-2 respectively. In vitro studies have indicated that N-demethylation may be an important metabolic pathway for HN-2 (1956).

2.3.2 Analytical Methods

Lemire et al. (2003, 2004) reported a quantitative method for the determination of *N*-ethyl- and *N*-methyldiethanolamine, the hydrolysis products of HN-1 and HN-2, in urine using LC-ES-MS-MS on a triple sector quadrupole instrument. The analytes were concentrated from urine by SPE on a strong cation exchanger. In order to obtain good peak shapes, 73% 3mM ammonium hydroxide (pH 10.5) - 27% methanol was used as the mobile phase for LC. Isotope dilution ([$^{13}C]_4$-*N*-Me and *N*-Et diethanolamines] was used to compensate for inherent variabilities. Detection was by MRM, monitoring the transition $MH^+ \rightarrow [MH\text{-}H_2O]^+$ for each analyte. The limits of detection were 0.4 ng/ml for *N*-ethyldiethanolamine and 1 ng/ml for *N*-methyldiethanolamine. An interferent was present in urine with similar retention time and nominal mass characteristics as *N*-methyldiethanolamine.

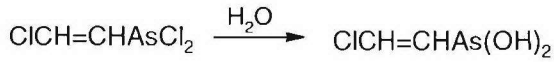
2.4 Lewisite

Like sulphur mustard, Lewisite is a potent vesicant but with greater volatility and a more rapid onset of action. Lewisite was first manufactured towards the end of the 1914-18 war, but not in time to be used in that conflict. Unlike sulphur mustard and nerve agents, no human exposures to Lewisite have been reported in which biomedical

samples have been collected. Weapon grade Lewisite is composed of ~90% Lewisite 1, $ClCH{=}CHAsCl_2$, up to ~10% Lewisite 2, $(ClCH{=}CH)_2AsCl$, and <1% of Lewisite 3, $(ClCH{=}CH)_3As$, all predominantly as the trans isomers. In addition to being weaponised neat, Lewisite was mixed with sulphur mustard to depress the freezing point of the latter and to impart a faster onset of effects. There remains a legacy of old and abandoned munitions, and former production sites.

2.4.1 *Metabolism*

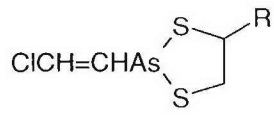
No detailed metabolism studies have been reported for Lewisite. It is rapidly hydrolysed to 2-chlorovinylarsonous acid (CVAA) (**13**) in the presence of moisture, and Waters and Williams (1950) reported that CVAA is excreted in the urine of experimental animals. This has since been confirmed by modern analytical methods.

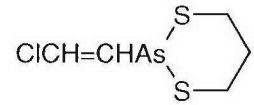


(13) CVAA

2.4.2 *Analytical Methods*

Trivalent arsenic forms strong bonds with sulphur, and thiols are therefore used for derivatising both Lewisite and CVAA, forming the same derivative (Black and Muir, 2003). Lewisite reacts with mono and dithiols, the reaction with dithiols ocurring rapidly at ambient temperature. In a competitive environment, Lewisite reacts almost exclusively with dithiols rather than monothiols (Haas, 1998). Three dithiols, 1,2-ethanedithiol, 1,3-propanedithiol and 2,3-dimercaptopropan-1-ol (British Anti-Lewisite, BAL) have been used for biomedical sample analysis of CVAA to form cyclic derivatives (**14a,b**) and (**15**). Unlike derivatisation of TDG, CVAA can be derivatised directly in aqueous solution. 2, 3-Dimercaptotoluene, which has been used extensively for environmental analysis (Black and Muir, 2003) does not appear to have been used.





(14a) R = H
(14b) R = CH_2OH

(15)

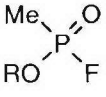
Jakubowski et al. (1993) developed a GC-MS method for CVAA spiked into guinea pig urine using 1, 2-ethanedithiol for derivatisation, with phenyl arsine oxide as internal standard. The same group later expanded the method to include atomic emission detection (Logan et al., 1999). CVAA was concentrated from urine (adjusted to pH 6 with 1 M HCl) by SPE on C18. After elution with methanol and concentration to dryness, the residue was reconstituted and derivatised with ethanolic 1, 2-ethanedithiol. Detection was by GC combined with arsenic selective AED and by EI-MS using SIM. Ions monitored were the moderately intense M^+ ion at *m/z* 228, an intense ion $[M\text{-}C_2H_4]^+$, *m/z* 200, and a base peak $[AsS_2C_2H_4]^+$, *m/z* 167. The LOD was rather high at ~100 ng/ml. CVAA was detected in the urine of guinea pigs up to 24 h after exposure to Lewisite (0.5 mg/kg s.c.). Excretion was rapid; a mean concentration of 3.5 ug/ml was detected in the 0-8 h urine, which had decreased to ≤ 100 ng/ml after 16-24 h.

Fidder et al. (2000) used BAL to derivatise free CVAA in blood and urine, and to displace Lewisite residues bound to a cysteine residue in haemoglobin (see Part 2). Blood or urine was incubated with BAL (ambient temperature, overnight), with phenylarsine oxide-BAL derivative as internal standard, and the dithiarsenoline derivative extracted using C18 SPE. The free hydroxyl on the resulting cyclic derivative (**14b**) was converted to its heptafluorobutyryl derivative with heptafluorobutyrylimidazole (50°C, 1 h, toluene); this was observable on the GC-MS chromatogram as a pair of diastereoisomers. This method was sensitive using GC-EI-MS SIM monitoring the molecular ion *m/z* 454 (20 pg injected); NICI was less suitable because of the absence of a pseudomolecular ion and the dominance of the non-specific $[C_3F_7CO_2]^-$ ion. The limit of detection was 10 nM (~2 ng/ml). CVAA could be detected in the urine of guinea pigs injected with 0.25 mg/kg Lewisite only up to 12 h post exposure.

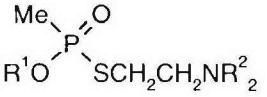
The most sensitive method for CVAA has recently been reported by Wooten et al. (2002) using solid phase microextraction to concentrate the derivatised analyte. Urine, with added ammonium acetate buffer and PhAsO as internal standard, was derivatised directly with 1, 3-propanethiol and the derivative concentrated on a poly(dimethylsiloxane) (PDMS) SPME fibre. Analysis was by automated GC-MS using selected ion monitoring of the isotopic MH^+ ions. An impressive detection limit of 7.4 pg/ml was reported, using a benchtop GC-MS system. The method was validated using spiked human urine.

2.5 Organophosphorus Nerve Agents

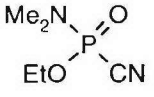
Organophosphorus nerve agents are the most potent of the lethal CW agents declared to the OPCW. They were developed just before, during and shortly after WW II, but not used in that conflict. Their use has since been confirmed in the Iraq-Iran conflict, against the Kurdish population in Iraq, and by terrorists in Japan. They can be divided into three structural classes (Figure 9): the phosphonofluoridates, usually referred to as G agents, e.g. sarin (GB), soman (GD), cyclosarin (GF); the V agents, VX (U.S.) and R-33 (Russia); and GA (tabun) (Black and Harrison, 1996).



sarin, GB, R =Me_2CH-
soman, GD, R = $Me_3CCH(Me)$-
cyclosarin, GF, R = c-C_6H_{12}



VX, R^1 = Et, R^2 = Me_2CH
RVX, R^1 = Me_2CHCH_2, R_2 = Et



tabun, GA

Figure 9 Chemical structures of nerve agents.

2.5.1 *Distribution and Metabolism*

The metabolism of nerve agents is much simpler than that of sulphur mustard. The major pathway for elimination is *via* enzyme mediated hydrolysis by phosphatases, plus some chemical hydrolysis, as shown in Figure 10. In the case of the methylphosphonofluoridates and V agents, the major product is an alkyl methylphosphonic acid (alkyl MPA) (**16**). A small fraction of the nerve agent binds covalently to cholinesterases, the biochemical target (see Part 2). Detailed elimination studies have been reported only for the phosphonofluoridates. Shih et al. (1994)

determined the elimination kinetics of the main metabolites of sarin, soman and cyclosarin in the rat (0.075 mg/kg, s.c.). The agents were initially distributed rapidly throughout the tissues. Urinary excretion, as the alkyl MPAs, was the major route of elimination for all three compounds. Only traces of methylphosphonic acid (MPA), from further hydrolysis of the acids, were observed, although significant amounts were reported in the urine/plasma of casualties of Japanese terrorist attacks with sarin (Nakajima et al., 1998; Minami et al., 1997). Approximately 90% of the applied dose of sarin and cyclosarin was excreted in the urine within 24 h, with terminal elimination half-lives of 3.7 h and 9.9 h respectively. Soman was excreted at a slower rate, approximately 50% within the first 24 h, rising to 62% after 7 days. It showed a biphasic elimination with elimination half-lives of 3.6 h and 18.5 h. Benschop et al. (1991) have shown that the first phase of elimination of soman is due to enzymatic hydrolysis of the inactive P(+) isomer, whereas the slower phase is from the active P(-) isomer. The alkyl MPAs were also detected in blood, isopropyl (iPrMPA) up to 14 h post exposure, cyclohexyl (cHexMPA) up to 2 days, and pinacolyl (PinMPA) up to at least 3 days (1994).

VX appears to follow a similar pathway, the major metabolite being ethyl MPA (EMPA). An additional metabolite, derived from the diisopropylaminoethyl substituent, was identified in human plasma following an assassination with VX (Tsuchihashi et al., 1998). The sulphide (**17**), derived from enzymatic S-methylation of the hydrolysis product $HSCH_2CH_2N(iPr)_2$, was identified in human serum by GC-MS after simple extraction. Experiments in rats confirmed the rapid metabolic formation of (**17**) from $HSCH_2CH_2N(iPr)_2$ (Tsuchihashi et al., 2000). Identification of this metabolite distinguishes VX from the *O*-ethyl analogue of sarin.

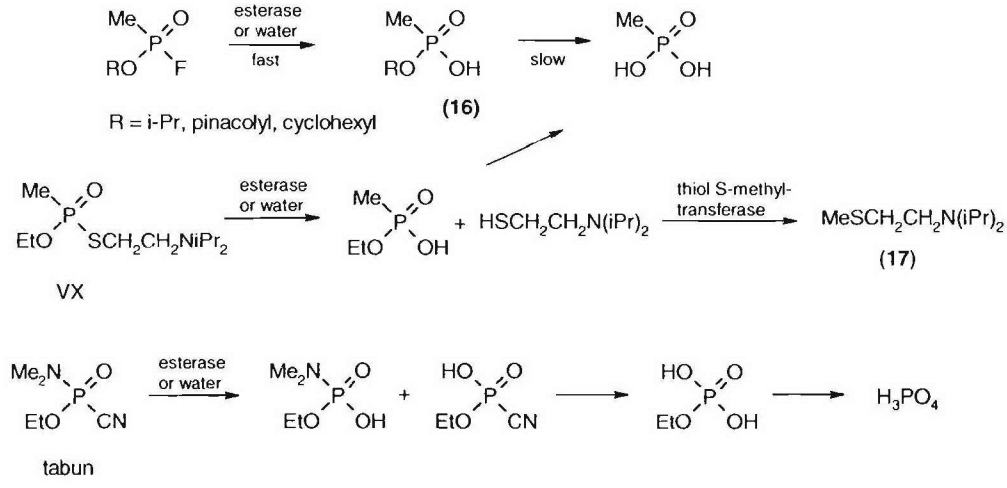


Figure 10 Metabolic and/or hydrolysis pathways for nerve agents.

2.5.2 *Analytical Methods for Nerve Agents and Their Metabolites*

2.5.2.1 *Unmetabolised Agent*

It is unlikely that unchanged nerve agent would be detected in the blood or tissues of a casualty unless samples were collected very soon after the exposure. A number of methods have been reported for the analysis of nerve agents in blood, for application to animal studies. These involve simple liquid or SPE extraction, e.g., using chloroform (sarin, soman) (Singh et al, 1985) C18 SPE (sarin, soman), (Benschop et al., 1985;

De Bisschop and Michiels, 1984), ethyl acetate (VX) (Bonnierbale et al., 1997) usually after precipitation of proteins, and analysis by GC-MS or GC-NPD. Sarin bound to cholinesterase and displaced with fluoride ion was extracted by C18 SPE (see Part 2) (Polhuijs et al., 1997).

2.5.2.2 Alkyl Methylphosphonic Acids

a) Overview

More than a dozen mass spectrometric methods have been reported for the trace analysis of alkyl MPAs in environmental and biomedical samples. Methods based on derivatisation and GC-MS-MS (or high resolution GC-MS) have provided the lowest limits of detection, although the latest generation of very sensitive LC-MS instruments may achieve comparable limits of detection (see an example below). TMS, TBDMS and methyl esters are commonly used for GC-MS analysis of alkyl MPAs in environmental samples (Black and Muir, 2003). Both types of silyl derivative were successfully used in the analysis of blood and urine samples from Japanese terrorists incidents, but many of the samples were collected within a few hours of the exposure. In cases of allegations of CW use, particularly in remote conflicts, samples are more likely to be collected many days after the alleged event, and much lower detection limits may be required. Another factor to consider is that casualties surviving an exposure to a nerve agent will probably have absorbed a much lower dose than the casualties who survived mustard poisoning, described in section 2.2.3. As is the case with TDG, conversion of alkyl MPAs to perfluorinated derivatives (pentafluorobenzyl esters) and analysis by NICI GC-MS-MS has provided the lowest limits of detection (Black and Muir, 2003).

Isolation from the matrix is a key stage in the analysis of alkyl MPAs and, in combination with subsequent derivatisation, is a potential source of error. A number of procedures that isolate the acids using SPE have been reported for blood and urine. These retain the ionised acid by anion exchange or the unionised acid by hydrophobic interactions on C_2, C_8 , C_{18} or polymeric cartridges. Alternatively, liquid-liquid extraction can be used. Ideally, a single procedure is desirable that efficiently recovers the range of alkyl MPAs of interest, but the widely differing hydrophobic properties, e.g. of EMPA and PinMPA, make this difficult. MPA is a particularly difficult acid to analyse at trace levels. An alternative to extracting the acids from the biological matrix is to remove as many extraneous materials as possible and then concentrate to dryness. Hydrophobic materials can be removed from urine at around neutral pH by elution through a C18 or polymeric cartridge, although losses of the more hydrophobic acids, PinMPA and cHexMPA, may occur. Inorganic cations or anions can be removed by selective ion exchange.

In our experience, GC-MS procedures for analysis of alkyl MPAs at the low ppb level lack robustness, possibly due to differences in SPE cartridges, the variability of urine, and inefficient derivatisation in the presence of extraneous materials. Most of the more sensitive methods have been developed for use with research grade mass spectrometers and transfer to more routine instruments may not be straightforward. A problem that occurs with the most sensitive methods is difficulty in achieving true blanks after analysing spiked samples.

b) GC-MS using pentafluorobenzyl esters

Several different procedures have been reported for the trace analysis of alkyl MPAs as their pentafluorobenzyl esters. Shih et al. (1991) isolated iPrMPA from acidified (pH 1)

urine or blood by SPE onto C18. The more hydrophobic PinMPA and cHexMPA were extracted onto C2. After washing with acidified sodium chloride solution, the acids were eluted with methanol. Potassium carbonate was added, the mixture concentrated to dryness, and derivatisation affected by treatment with pentafluorobenzyl bromide (PFBBr) in dichloromethane using 18-crown-6 ether as catalyst (50°C, 1 h). $C[^2H]_3$-P alkyl MPAs were used as internal standards. GC-MS analysis was performed using EI, positive ion CI and negative ion CI, limits of quantitation being 1 ng/ml for PinMPA, 5 ng/ml for cHexMPA and 10 ng/ml for iPrMPA. Fredriksson et al. (1995) reported a similar method but extended it to include other alkyl MPAs including EMPA, and again incorporating deuterated acids as internal standards. A strong anion exchange (SAX) resin was used to capture ionised alkyl MPAs, followed by elution with 0.3 M sodium bromide solution. After concentration to dryness, derivatisation was performed with PFBBr and potassium carbonate in acetonitrile (90°C, 1 h). The derivatised acids were further cleaned up on a Florisil cartridge. A low and variable recovery of the bis-PFB ester of MPA was noted. Analysis was by GC-MS-MS using a hybrid magnetic sector - quadrupole tandem mass spectrometer at 1000 resolution of MS1 and unit resolution of MS2. CID was performed at a high collision energy (80-100 eV) in order to produce several structurally specific product ions in addition to the major one resulting from loss of alkene from $[M\text{-}PFB]^-$. The method was inherently extremely sensitive, low attogram amounts being detectable in standards, but limits of detection in urine and plasma were not quoted.

Both of these procedures involve a number of manipulations, including concentration to dryness before derivatisation, and a heterogeneous derivatisation procedure in the presence of quite large amounts of inorganic salts. A simplified, though still quite involved procedure, uses a polymeric cartridge for extraction of acidified urine, acetonitrile for elution and derivatisation either after concentration to dryness or directly in the eluate (Riches et al., 2005). The derivatised solution is cleaned up on Florisil and analysed by NICI GC-MS-MS after the method of Fredriksson. Although optimum sensitivity (~0.1 ng/ml) was achieved using an expensive hybrid magnetic sector quadrupole instrument, limits of detection in the range 0.5 -1 ng/ml have been achieved using a relatively inexpensive benchtop ion trap MS. However, only one significant product ion, due to loss of alkene from $[M\text{-}PFB]^-$, is obtained from the lower energy CID used in this system. Chromatograms from urine spiked at 1 ng/ml are shown in Figure 11. It should be noted that extraction efficiency is low (10-15%) for the least hydrophobic acid EMPA, and if this were the main analyte of concern then an alternative extraction procedure should be used.

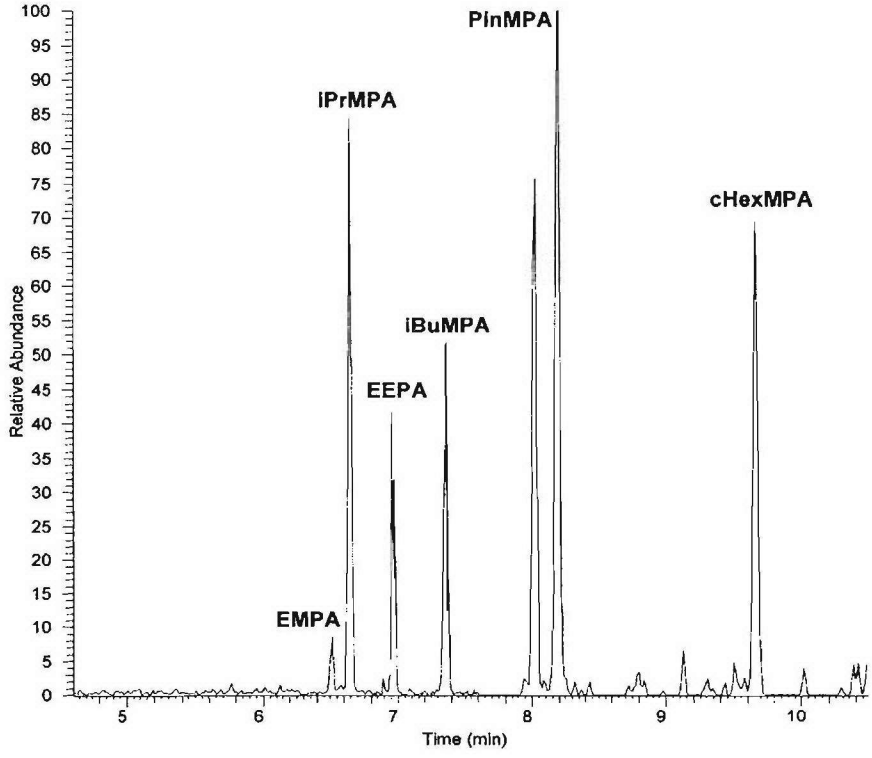


Figure 11 GC-MS-MS-MRM chromatograms showing the detection of alkyl MPAs, spiked into human urine at 1 ng/ml, as pentafluorobenzyl esters NICI and a benchtop ion trap instrument.

Miki et al. (1999) have reported a modification to the derivatisation procedure, adapted from organophosphorus pesticide residue analysis, in which derivatisation and concentration of the analytes are combined. Urine samples, with dipropyl phosphoric acid added as internal standard, were passed through a cation exchange cartridge (Ag^+ form) to remove chloride ions, and the pH of the eluate adjusted to 4.5. Derivatisation was performed under phase transfer conditions consisting of aqueous eluate with added phosphate buffer, PFBBr in toluene, and a polymer-bound quaternary phosphonium phase transfer catalyst (tri-n-butylmethylphosphonium bromide) (vigorously stirred, 85°C, 90 min). The organic fraction was cleaned up on Florisil. This method avoids the need to concentrate an aqueous eluate to dryness, and results in a lower inorganic salt content in concentrates; however, it is experimentally complex. Detection limits were in the range 2.5-10 ng/ml using GC-MS-EI (SIM) and 0.06 ng/ml using GC-MS-NICI, although the chromatograms at 1 ng/ml showed a number of additional components (these would no doubt be much cleaner using MS-MS). The procedure could also be applied to deproteinated serum and diluted saliva.

The pentafluorobenzyl esters generally give sharp, symmetrical GC peaks. They are usually intended specifically for use with NICI-MS. With positive EI, much of the ion current is concentrated in the non-specific ion *m/z* 181, $[C_6F_5CH_2]^+$ A moderately intense class specific ion is present at *m/z* 256, $[M\text{-}R\text{-}F]^+$, from loss of the alkyl group and a fluorine. Intense MH^+ ions are observed with positive isobutane CI. With NICI, the base peak is $[M\text{-}\ C_6F_5CH_2]^-$; the base peak is thus the anion of the acid. With virtually all the ion current concentrated in this ion, very high sensitivity can be obtained using SIM. Signal to noise ratios are further enhanced using MS-MS, monitoring the transition $[M\text{-}C_6F_5CH_2]^- \rightarrow [M\text{-}C_6F_5CH_2\text{-}C_nH_{2n}]^-$ ($[M\text{-}C_6F_5CH_2\text{-}C_6H_{10}]^-$

in the case of cHexMPA). A disadvantage is that these are the only ions or fragmentations suitable for monitoring with most mass spectrometers where low collision energy is used for CID.

c) GC-MS using methyl esters
Driskell et al. (2002) developed a method that detected alkyl MPAs in urine as their methyl esters. Urine was simply concentrated to dryness by azeotropic distillation of water with acetonitrile, and the residue derivatised with ethereal diazomethane (10 min, ambient temperature). The method was designed for high throughput rather than low limits of detection (4 ng/ml for alkyl MPAs, 20 ng/ml for ethyl phosphoric acid). Recoveries of the acids from GB, GD, GF, VX and GA were within the range 30-50%. Analysis was performed by GC-MS-MS on a triple sector quadrupole instrument, using positive ion CI with isobutane as reagent gas. Ions monitored were $MH^+ \rightarrow [MH-C_nH_{2n}]^+$ ($[MH-C_6H_{10}]^+$ with cHexMPA) for the five acids. Deuterated acids were used as internal standards. Methyl esters have less than ideal properties for trace analysis. They can give rise to poor peak shapes, have relatively short retention times, and the highest mass ions are usually below *m/z* 200 and prone to interferences using SIM. Recently, this method was optimized, obtaining detection limits < 1 ng/ml (Barr et al., 2004).

d) GC-MS/GC-FPD using silyl esters
The most widely used procedures for environmental analysis of alkyl MPAs use conversion to trimethylsilyl (TMS) or *tert.*-butyldimethylsilyl (TBDMS) esters. Both have also been applied to samples associated with terrorist use of sarin or VX in Japan, although they are less sensitive than methods employing pentafluorobenzyl esters. Derivatisation conditions for most applications typically involve treatment with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) +/- 1% trimethylsilyl chloride (TMSCl) (60°C, 30 min) or *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide MTBSTFA + 1% *tert.*-butyldimethylsilyl chloride (TBDMSCl) (60°C, 30-60 min).

Two methods adopted the approach of removing extraneous materials from urine and concentration to dryness prior to derivatisation. Minami et al. (1997) used a 3 layered cation exchange resin loaded with Ag^+, Ba^{++} and H^+ to remove chloride ion, sulphate ion and pigments respectively. After adjustment of the pH of the eluant to ~3.7, the solution was lyophilised before treatment with BSTFA + 10% TMCS. LODs using GC-FPD were 25 ng/ml for iPrMPA and EMPA, rather higher for MPA. Nakajima et al. (1998) passed urine through C18 to remove hydrophobic material and then a cation exchange resin (H^+ form) to remove metal ions. After concentration to dryness, the residue was reconstituted in tetrahydrofuran and derivatised with MTBSTFA in pyridine, NaCl added, and the derivative partitioned into hexane containing p-cyanophenyl dimethyphosphate as internal standard. Using GC-FPD, the LOD for MPA was 10 ng/ml (LOD for iPrMPA not quoted).

Katagi et al. (1997) detected EMPA in serum as its TBDMS derivative. Serum was deproteinated by micro-ultrafiltration and extracted with dichloromethane for unmetabolised VX [and VX metabolite (**17**)]. The aqueous fraction was acidified with oxalate buffer (pH 1.68), sodium chloride added to salt out the analyte, and EMPA extracted into acetonitrile. After concentration to dryness, the residue was derivatised with MTBSTFA + 1% TBDMSCl (60°C, 30 min). The LOD by GC-EI-MS was 3 ng/ml monitoring the most intense ion (*m/z* 153), 40 ng/ml using full scan EI, and 80 ng/ml using full scan isobutane CI.

e) LC-MS-MS

Although LC-MS has generally been less sensitive than GC-MS for alkyl MPAs (Read and Black, 1999), the latest LC-MS-MS instrumentation is enabling lower LODs to be achieved. Noort et al. (1998) reported a sensitive micro-LC-MS-MS method for the detection of iPrMPA in serum. C[^{2}H]$_3$-iPrMPA was added as internal standard, the solution acidified with dilute sulphuric acid, and iPrMPA extracted with isobutanol/toluene 1:1. A microbore PRP-X100 (anion exchange) column was used for LC, isocratically eluted with H_2O/CH_3CN 1:1 plus 0.5% formic acid. The LOD was 1-4 ng/ml using either positive or negative ion ESI-MS-MS. Ions monitored were $[M\text{-}H]^-$ $\rightarrow$ $[M\text{-}H\text{-}C_3H_6]^-$ (*m/z* 137$\rightarrow$95) in negative mode and $MH^+ \rightarrow [MH\text{-}C_3H_6]^+$ (*m/z* 139$\rightarrow$97) in positive mode (plus the corresponding ions from the internal standard). The same technique was also applied for the detection of pinacolylmethylphosphonic acid (PMPA), the hydrolysis product of soman, in urine of rhesus monkeys that had received a sign-free dose of soman (Van der Schans et al., unpublished results). However, the extraction was now performed with a mixture of ether and acetonitrile (v/v 85:15). The lowest detectable concentration was 0.2 ng/ml in urine. Figure 12 shows the excretion pattern of PMPA in urine of a rhesus monkey that was injected with a sign free dose of nerve agent (0.4 µg/kg), adjusted to reach 30% BuChE inhibition. PMPA could be detected up to 6 days after injection of the agent

The method was applied successfully to human samples (see below).

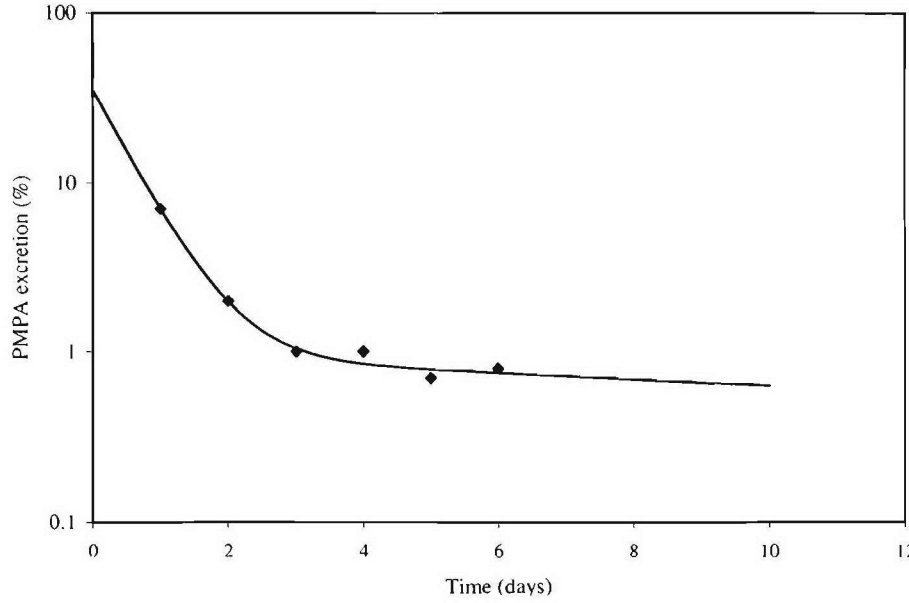


Figure 12 Pinacolyl-MPA in urine of rhesus monkey that had received a sign-free dose of soman (i.v.) adjusted to obtain 30% BuChE inhibition.

2.5.3 *Detection in Cases of Human Poisoning*

The only reported incidents of nerve agent poisoning, where biomedical samples have been obtained, are those resulting from terrorist dissemination of sarin in Matsumoto (1994) and the Tokyo subway (1995), plus an assassination using VX, also in Japan (Tu, 2002). In contrast to the CW incidents involving sulphur mustard, many of the biomedical samples associated with these terrorist attacks were collected within hours of the event.

Very high levels of MPA and iPrMPA were detected as their TBDMS derivatives by GC-FPD in the urine of a Matsumoto casualty rendered unconscious and with low blood AChE activity (Nakajima et al., 1998). Urine was collected over a 7 day period. Concentrations of iPrMPA and MPA respectively were: day 1, 760 ng/ml and 140 ng/ml; day 3, 80 ng/ml and 20 ng/ml; day 7, 10 ng/ml, MPA not detected (no standard of iPrMPA was available and quantitation was based on detector response to MPA). The exposure was estimated as 2.79 mg of sarin, making crude assumptions on the percentage that would be excreted. iPrMPA was detected as the TMS derivative by GC-FPD in urine collected over 7 days from casualties of the Tokyo attack (Minami et al, 1997). Concentrations were not reported but the estimated exposures were 0.13-0.25 mg of sarin in a comatose patient and 0.016-0.032 mg in less severely intoxicated patients. Using LC-MS-MS and a more rigorous method of quantitation, iPrMPA was detected underivatised in serum at concentrations of 3-136 ng/ml in 4 casualties of the Matsumoto incident and 2-100 ng/ml in 13 casualties of the Tokyo attack (Noort et al., 1998). All samples were taken within 2.5 hours of hospitalisation. High levels of iPrMPA correlated with low levels of butyrylcholinesterase activity. Other positive analyses associated with these incidents were obtained by identification of inhibited cholinesterase and are reported in Part 2.

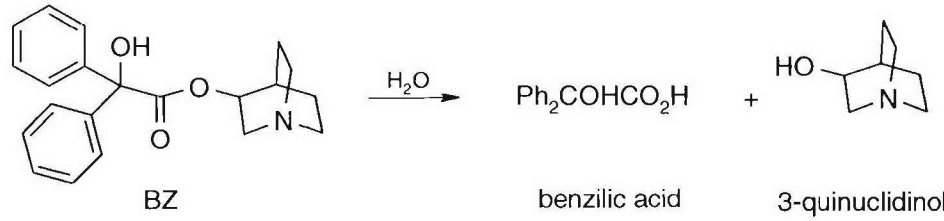
EMPA was detected as its TBDMS derivative by GC-MS and GC-MS-MS in the serum of a subject assassinated by application of VX from a disposable syringe to the neck (Tsuchihashi et al., 2000). A concentration estimated as 1250 ng/ml was detected in serum from blood collected 1 h after the exposure and stored at -20°C for several months. 2-(Diisopropylaminoethyl) methyl sulphide (**17**) was also detected at an estimated concentration of 143 ng/ml.

2.6 3-Quinuclidinyl Benzilate, BZ

BZ is an incapacitating agent that acts on the central nervous system, by reversible blockade of muscarinic-type receptors in the cholinergic nervous system. Incapacitating doses are in the low microgram per kilogram range. Mydriasis is one overt symptom indicating possible exposure. Unlike the vesicants and nerve agents, BZ is not known to form covalent bonds with glutathione or nucleophilic sites on macromolecules. It has been much less of a concern as a CW agent than nerve agents and vesicants, and this is reflected in the paucity of methods for the analysis of biomedical samples.

2.6.1 Metabolism

The metabolism of BZ appears to be dominated by simple hydrolysis to benzilic acid and 3-quinuclidinol.



BZ benzilic acid 3-quinuclidinol

2.6.2 Analytical Methods

BZ and 3-quinuclidinol can be analysed underivatised by GC-MS or LC-MS, but retention times for BZ are long, sensitivity is modest and it tends to decompose to benzophenone on hot surfaces. Byrd et al. (1987, 1988) described a GC-MS method

using TMS derivatives for the determination of BZ and its hydrolysis products in urine. Each analyte was isolated from urine using separate SPE extraction procedures. BZ and benzilic acid were isolated on C18 after basification and acidification of urine respectively. Quinuclidinol was more difficult to isolate; recoveries of 40-60% were obtained at pH 6-7 using Florisil SPE. Concentrated residues were derivatised with *N*-methyl-*N*-trimethysilyltrifluoroacetamide (MSTFA) in acetonitrile (3 h, 70°C for BZ, 15 min ambient temperature for benzilic acid and 3-quinuclidinol). The method used isotope dilution, requiring ^{18}O-quinuclidinol, $^{2}H_5$-benzilic acid, and ^{18}O, $^{2}H_5$-BZ. Urine was treated with glucuronidase prior to analysis although there is no information on the formation of glucuronides. Neither BZ-TMS nor benzilic acid TMS give significant M^+ ions in their EI mass spectra, but share a common base peak $[Ph_2COTMS]^+$, *m/z* 255 suitable for SIM. Quinuclidinol-TMS gives a moderately strong (50%) M^+, *m/z* 199 suitable for SIM. Both BZ-TMS and quinuclidinol-TMS tend to exhibit peak tailing on low polarity GC columns. The method could detect BZ at concentrations of 0.5 ng/ml and the hydrolysis products at 5 ng/ml, using large (20 ml) aliquots of urine for each analyte. Benzilic acid also forms a TBDMS derivative but this has not been applied to biomedical sample analysis.

2.7 Phosgene

Phosgene was used as a major CW agent in WW I and was responsible for the greatest number of CW induced mortalities. Currently, it is a major industrial chemical. It causes a lethal pulmonary oedema after an asymptomatic lag phase of up to 24 h. Its specific mechanism of action is unknown but is assumed to involve reactions with nucleophilic sites on macromolecules.

2.7.1 Metabolism

Aspects of phosgene metabolism have been periodically reported, particularly as an active metabolite of chloroform. It will react with two molecules of glutathione to form a bis conjugate (Fabrizi et al, 2001) and with cysteine to form 2-oxothiazolidine-4-carboxylic acid (Kubic and Anders, 1980), but it does not appear to have been established if significant amounts of these or related compounds are excreted in urine. As an active metabolite of chloroform, phosgene reacts with the polar heads of phospholipids (Di Consiglio et al., 2001). Its reactions with blood proteins are described in Part 2.

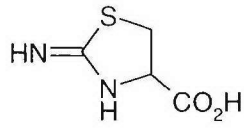
2.8 Hydrogen Cyanide

Like phosgene, hydrogen cyanide (HCN) was used in WW I, but on a smaller scale and with limited effect. It is produced in thousands of tonnes as a legitimate industrial chemical and fumigant. Apart from its status as a CW agent, plus its occasional use in human poisoning, HCN is a toxic component of smoke produced by cigarettes and fires. HCN is known as a lethal 'blood' gas. It reacts with trivalent iron of cytochrome oxidase, thereby inhibiting aerobic cell metabolism and starving the blood of oxygen.

2.8.1 Metabolism

HCN can react with both nucleophiles and electrophiles, depending on the conditions. Most of the cyanide in blood concentrates in the red cells bound to methemoglobin, which acts as a cyanide sink. It can be released by heating with acid. Cyanide is metabolised to thiocyanate (SCN), through the action of a sulphotransferase enzyme called rhodanese, located mainly in the liver. Cyanide is excreted primarily as

thiocyanate in urine. Approximately 15% of absorbed cyanide reacts with cystine to form iminothiazolidine carboxylic acid (**18**). Cyanide also reacts with vitamin B12 and appears as cyanocobalamin in urine (Borowitz et al., 1992).



(18)

2.8.2 Analytical Methods

2.8.2.1 Overview

Numerous methods have been reported for the analysis of cyanide and thiocyanate in biomedical samples, mostly for the determination of cyanide levels in smokers and fire victims rather than cases of deliberate poisoning. These methods include visible, UV and fluorescence based spectrometric methods, electrochemical methods using ion selective electrodes, and GC with NPD, ECD or MS. It is not intended to cover all of these but focus mainly on chromatographic methods.

2.8.2.2 U.S. Army Method for Cyanide

The U.S. Army Medical Research Institute of Chemical Defence employs an automated micro-distillation assay for cyanide in blood, using fluorometric detection (Anonymous, 1996). For assaying free CN^-, plasma is mixed with and dialysed against pH 7.4 phosphate buffer. For assay of total blood cyanide, blood is mixed with saline containing Triton X (which haemolizes the red cells), treated with 0.5% sulphuric acid and dialysed against 0.25% sulphuric acid. Cyanide is isolated by microdistillation and assayed fluorometrically. The automated procedure takes approximately 16 min.

2.8.2.3 GC and GC-MS methods for Cyanide and Thiocyanate

A number of strategies have been employed for GC determination of cyanide and thiocyanate in blood. Major ones are the direct analysis of cyanide in the headspace, conversion of cyanide and thiocyanate to cyanogen chloride, and derivatisation to pentafluorobenzyl cyanide and pentafluorothiocyanate. Selected recent examples are reported below.

Methods based on headspace analysis provide low limits of detection. Typically, blood is incubated for up to ~30 minutes with phosphoric acid at temperatures between 50°C and 70°C. Ascorbic acid may be added to blood to prevent the conversion of thiocyanate to cyanide (Seto, 1996); acetonitrile or propionitrile may be added as internal standards. Cryogenic oven trapping of cyanide from the headspace combined with GC-NPD analysis provided a detection limit of 2 ng/ml (Ishii et al., 1998). Alternatively, solid phase microextraction of the headspace (using a Carbowax / divinylbenzene coated fibre) gave a detection limit of 20 ng/ml (Takekawa et al., 1998) Calafat and Stanfill (2002) reported an experimentally simple high throughput method using a fully automated headspace GC-NPD system, which provided a detection limit of 14 ng/ml. After addition of ascorbic acid, cyanide was liberated by heating blood with phosphoric acid (60°C, 5 min). The assay was validated on samples from fire victims.

In an alternative approach, cyanide and thiocyanate can be converted to cyanogen chloride using the chlorinating agent chloramine T. Conversion can be performed in

solution or in the headspace. Conversion in solution followed by headspace analysis gave a detection limit of 5 ng/ml by GC-ECD (Odoul et al., 1994). Conversion in the headspace above acidified blood, in a precolumn packed with chloramine T powder and attached to the injection port of the GC, gave a detection limit of 50 ng/ml (Maseda et al., 1989).

Derivatisation to pentafluorobenzyl cyanide provides an alternative and sensitive assay for both cyanide and thiocyanate, and allows sensitive detection by ECD, or by MS using SIM at a much higher mass than the native molecule. Chen et al. (Chen et al., 1994) reported a two phase derivatisation of several anions, including cyanide and thiocyanate, in potassium borate buffer (pH 9.5) with PFBBr in dichloromethane, plus an immobilised phase transfer catalyst (Kryptofix 222 B polmer). Using GC-ECD, sub-nanomolar concentrations of cyanide could be detected in urine and saliva. Kage et al. (1996) analysed cyanide and thiocyanate in blood using a two-phase pentafluorobenzylation procedure with PFBBr and tetradecyldimethylbenzylammonium chloride as phase-transfer agent. For cyanide determination, blood was pre-treated with sodium sulphite to prevent oxidation of blood cyanide and with trichloroacetic acid to precipitate proteins. Thiocyanate in blood was determined without deproteination. GC-ECD was used for quantitative analysis and GC-MS for qualitative analysis. Detection limits for cyanide and thiocyanate using ECD were 0.01 and 0.003 µmol/ml blood respectively. The EI mass spectrum of pentafluorobenzyl cyanide gave a molecular ion (*m/z* 207) as the base peak; pentafluorobenzylthiocyanate gave a weak M^+ and a base peak $[M\text{-}SCN]^+$ at *m/z* 181.

2.8.2.4 Iminothiazolidine carboxylic acid

Lundquist et al. (1995) reported a method for the analysis of the metabolite 2-aminothiazoline-4-carboxylic acid (**18**) in urine using LC with fluorescence detection after conversion to *N*-carbamylcysteine by heating with alkali. The analyte was concentrated from urine by cation exchange resin and further processed to remove interfering thiols and disulphides. The LOD was rather high at 0.3 µM.

2.8.3 Application to Human Casualties

Cyanide and thiocyanate are normal constituents of blood. Sources of cyanide include some foods, e.g., cyanogenic glycosides in bitter almonds, fruit seeds, and a number of plants, cigarette smoke, and smoke from fires. The blood concentration of cyanide in healthy subjects was 8.4 ± 3.1 ng/ml by the method of Ishii et al. (1998); in nine fire victims concentrations determined were 687 ± 597 ng/ml. In smokers, cyanide levels in blood may rise to ~500 ng/ml.

Kage et al. (1996) applied their GC-ECD/GC-MS method to the analysis of blood levels in two casualties who died from cyanide poisoning. Blood levels of cyanide and thiocyanate in a subject who died following injestion of sodium cyanide were 0.52 and 0.10 umol/ml respectively (= ~13 and 5.6 µg/ml). Levels determined in a fatal victim of smoke inhalation were 0.28 and 0.13 umol respectively (= ~7.3 and 7.5 µg/ml).
Fatal levels of cyanide are estimated as ~ 0.05-0.1 umol/ml (= ~1.3-2.6 µg/ml).
The lower levels of thiocyanate in comparison to cyanide in the blood were attributed to the sudden death; they were twice the mean levels (~0.06 µmol/ml) found in cigarette smokers. 2-Aminothiazoline-4-carboxylic acid was detected in the urine of moderate cigarette smokers at concentrations between <0.3 -1.1 µM (Lundquist et al., 1995).

3 Part 2 Analysis of adducts of chemical warfare agents with macromolecules

3.1 General

The major disadvantage of free metabolites as biological indicators is their relatively rapid elimination from the body, as was discussed in Part 1 of this report. Experience has shown that only ultra trace levels are likely to be detected if urine is collected > ~2 weeks after the exposure. Depending on the scenario and application, it is clearly advantageous to have biological indicators that are more slowly eliminated, ideally allowing retrospective identification up to several months following an exposure. Covalent adducts with macromolecules, such as proteins and DNA, offer potentially much longer-lived biological indicators of exposure in comparison to free metabolites. Part 2 of the report presents an overview of adducts that have been identified for CW agents, and the methods currently available for their detection. Applications to cases of human exposure are reported.

3.1.1 DNA and Protein Adducts

3.1.1.1 DNA Adducts

Vesicants, nerve agents and phosgene are reactive electrophiles that react covalently with nucleophilic sites on macromolecules. Reactive nucleophilic sites exist on the bases and phosphate groups of DNA molecules. An advantage of DNA as a substrate is that it is present in all tissues of the body. A disadvantage is that repair mechanisms tend to excise the alkylated moiety, resulting in a much shorter lifetime compared to alkylated proteins (for a recent review of mass spectrometry for quantitation of DNA adducts see Koc and Swenberg (2002).

3.1.1.2 Protein Adducts

Several of the constituent amino acids of proteins contain nucleophilic groups; examples are cysteine (SH), serine, tyrosine (OH), lysine (NH_2), and aspartic and glutamic acids (CO_2H). Covalent adducts with proteins offer potentially long lived biological indicators of exposure, provided they survive the normal lifetime of the macromolecule. Particularly useful as biological indicators are adducts with the abundant blood proteins hemoglobin and albumin (for recent reviews see Törnqvist et al., 2002 and Boogaard, 2002). These two proteins normally interact by purely chemical reactions of peripheral amino acid residues with the electrophile. Measurement of hemoglobin adducts is a well-established method for biomonitoring of environmental or occupational exposure to alkylating carcinogens. Hemoglobin adducts are generally stable (in contrast to alkylated DNA) and most appear to have lifetimes in humans similar to the native protein (approximately 120 days). Consequently, the adducts may be detectable for a long period after the actual exposure. In case of chronic exposures, the adducts will accumulate in time. Hemoglobin is abundant in human blood (140 mg/ml) and can be readily isolated as globin. Albumin is also a very abundant protein present in blood (30-45 mg/ml) but has a shorter lifetime than hemoglobin (half-life in humans 20 days). In the case of nerve agents, a selective catalytic reaction with two less abundant proteins, acetyl- and butyrylcholinesterase, provides very specific biological indicators of exposure.

3.2 Analytical Methods

The analysis of adducts with macromolecules can be more demanding than the analysis of free metabolites. In the case of non-catalysed adduct formation, only a small fraction of the available macromolecule reacts with the CW agent, and the adduct must therefore be detected against a heavy background of native macromolecule. DNA adducts can be detected by mass spectrometric assays after enzymatic or chemical hydrolysis to alkylated nucleotides, nucleosides or bases (Koc and Swenberg, 2002). Other techniques, such as ^{32}P-postlabeling and immunoassays, are also applicable but require mass spectrometric confirmation in the case of forensic samples. A number of different strategies have been applied to the mass spectrometric analysis of protein adducts. One approach is a non-selective enzymatic or acidic digestion of the protein to the individual amino acids, with detection of the discrete amino acid adduct. This approach is advantageous in certain cases but the chemical background produced can be very high. An exception is the selective cleavage of alkylated N-terminal valine on hemoglobin. Alternatively, a more selective enzymatic digestion can be used, e.g., with trypsin, to provide a small alkylated or phosphylated peptide. Both of these methods may require demanding sample preparation procedures, usually combined with LC/electrospray tandem MS (LC-MS-MS) analysis. GC-MS-MS is applicable to some amino acid adducts. Both approaches may be complicated by steric hindrance of the digestive enzyme by the bound agent residue, e.g., in some cases the enzyme Pronase may not efficiently digest the protein to its individual amino acids. A third approach involves chemical displacement of the covalently bound CW agent residue. This provides the original CW agent, a related compound, or its hydrolysis product, which is analysed in the same manner as a free metabolite. The advantage of this approach is that sample preparation may be simpler, and analysis performed using GC-MS or GC-MS-MS. Immunoassays provide complementary methodology for screening and monitoring purposes.

3.3 Sulfur mustard

Sulfur mustard remains one of the CW agents of greatest concern because of its ease of production, favorable physicochemical properties and potent vesicant action. It is a bifunctional alkylating agent, which reacts rapidly under physiological conditions with nucleophilic sites in proteins and DNA to form covalent adducts, via an intermediate episulfonium ion (see Figure 13). In the sections below the various adducts (as unambiguously elucidated in recent years by mass spectrometry) are addressed, and methods for their analysis are discussed.

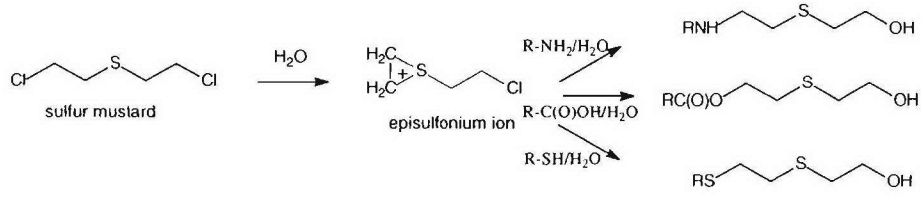


Figure 13 Sulfur mustard adduct formation with various nucleophiles.

3.3.1 Sulfur Mustard Adducts with DNA

3.3.1.1 Sites of Reaction

By analogy to several nitrogen mustard-based anti-tumor agents, the primary site of DNA alkylation by sulfur mustard is the N7 position of deoxyguanosine residues (Brookes and Lawley, 1960; Fidder et al., 1994).
Upon depurination of the resulting N7-(2-hydroxyethylthioethyl)-2'-deoxyguanosine, N7-(2-hydroxyethylthioethyl)guanine (N7-HETE-Gua) is obtained (see Figure 14). Minor amounts of the N7-guanine di-adduct and the N3-adenine adduct were also detected.

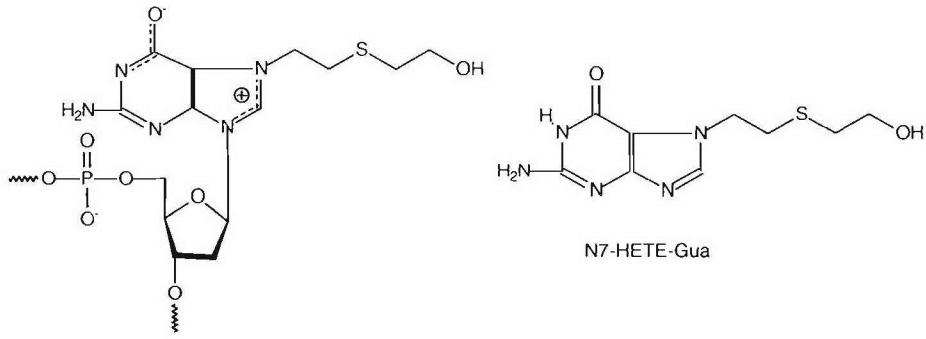


Figure 14 N7-Deoxyguanosine adduct of sulfur mustard and derived N7-(2-hydroxyethylthioethyl)guanine (N7-HETE-Gua).

3.3.1.2 Analytical Methods

a) Mass spectrometric
GC-MS analysis of (N7-HETE-Gua) proved problematic. Derivatization with heptafluorobutyric acid anhydride and pentafluorobenzyl bromide was troublesome and silylation afforded a derivative with poor gas chromatographic properties. The underivatized compound could however be conveniently analyzed in urine using LC/electrospray tandem MS, using a C18 cartridge for extraction (Fidder et al., 1996). The adduct was readily detected in the urine of guinea pigs exposed to sulfur mustard, although levels dropped rapidly after 36-48 h, and after processing of skin and blood samples of animals exposed to sulfur mustard. Using similar methodology, N7-HETE-Gua was detected in the spleen and liver of rats exposed percutaneously to sulfur mustard (Rao et al., 2002).

b) Other physicochemical methods
The group of Ludlum developed an HPLC method for analysis of N7-HETE-Gua (Ludlum et al., 1994) and also a ^{32}P-postlabeling method for N7-HETE-deoxyguanosine 5'-phosphate (Niu et al., 1996).

c) Immunoassay
An ELISA was developed by Van der Schans et al (1994; 2004) for detection of the mustard adduct within DNA, using monoclonal antibodies raised against N7-HETE-guanosine-5'-phosphate coupled to keyhole limpet hemocyanin The ELISA was successfully applied in toxicokinetic studies in which levels of adducted DNA were followed in conjunction with measurement of intact sulfur mustard (Langenberg et al., 1998).

3.3.1.3 Application to Human Casualties

The ELISA for detection of the DNA adduct was successfully applied to blood samples from two casualties of the Iraq-Iran conflict. These samples were collected 22 and 26 days following the alleged exposure to sulfur mustard (Benschop et al, 1997). Concentrations found in lymphocytes and granulocytes were equivalent to similar levels found in human blood after treatment *in vitro* with 0.015-0.43 μM sulfur mustard.

3.3.2 Sulfur Mustard Adducts with Hemoglobin

3.3.2.1 Sites of Reaction

Distribution studies of ^{35}S-sulfur mustard in rats after cutaneous exposure to sulfur mustard, and human blood treated *in vitro,* showed that a small percentage of radioactivity remained associated with the hemoglobin and persisted for the lifetime of the red cells (Hambrook et al., 1993). The alkylation of proteins by sulfur mustard was studied in the 1940s and 1950s by various groups (for a review see Wheeler, 1962), indicating that alkylation of, *e.g.*, carboxyl, amino and sulfhydryl groups, readily occurs. Definitive evidence for specific alkylation sites has been obtained more recently by using modern mass spectrometric techniques. LC/electrospray tandem MS analysis of tryptic digests, of hemoglobin treated with radiolabelled sulfur mustard, identified alkylation on 6 different histidine residues, 3 glutamic acid residues, and both of the N-terminal valines (Noort et al., 1996; Black et al., 1997) most of these residues are peripherally located, as should be expected. Alkylated cysteine, aspartic acid, lysine and tryptophan were also detected in Pronase digests. The N1 and N3 histidine adducts were found to be the most abundant adducts.

3.3.2.2 Analytical Methods

Although the degree of alkylation of the N-terminal valine in human hemoglobin is only 1-2% of the total alkylation induced in hemoglobin upon treatment of human blood with sulfur mustard, N-alkylated N-terminal valine has the advantage as a biomarker of exposure that it can be selectively cleaved from hemoglobin by a modified Edman procedure. This method, using pentafluorophenyl isothiocyanate as reagent, was originally reported by Törnqvist *et al.* (1996) (see Figure 15) for other alkylating agents. Globin is isolated from human blood using standard procedures, dissolved in formamide, and treated with the reagent at 45°C for 2 h. The derivatised N-alkylated valine is extracted into diethyl ether, the solvent removed and the residue redissolved in toluene. Analysis of the resultant pentafluorophenyl thiohydantoin, using negative ion GC-MS-MS after further derivatization with heptafluorobutyric anhydride, provided a sensitive method for the detection of the N-alkylated valine (Fidder et al., 1996; Black et al, 1997). The lowest detectable exposure level of human blood *in vitro* was 0.1 μM (Noort et al., 2004). An *in vivo* study with marmosets demonstrated the potential for hemoglobin adducts as relatively long-lived biological markers of sulfur mustard poisoning (Benschop et al, 2000). After administration of a single dose of sulfur mustard (4.1 mg/kg) the N-terminal valine adduct was still detectable after 94 days (see Figure 16). Remarkably, the adduct level increased for several days after administration of sulfur mustard, which indicates a pronounced stability of the intact agent.

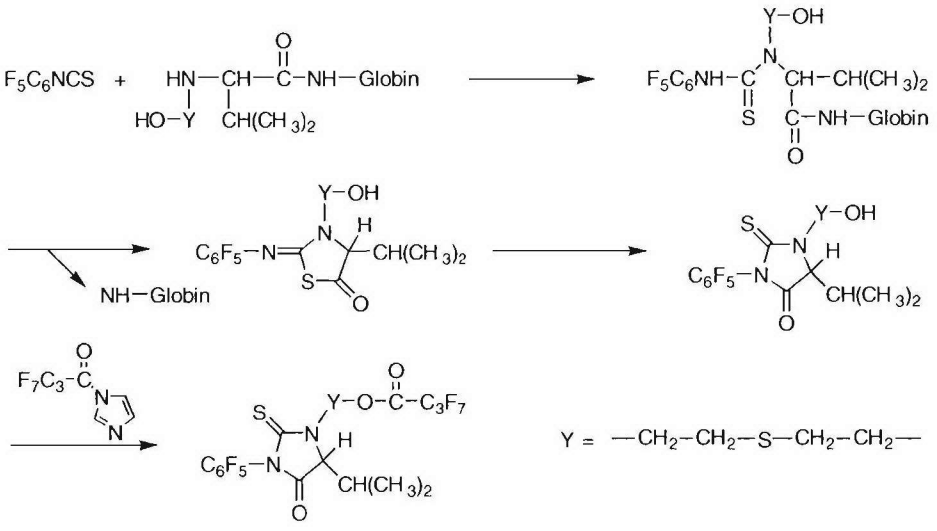


Figure 15 Modified Edman degradation procedure of globin alkylated by sulfur mustard.

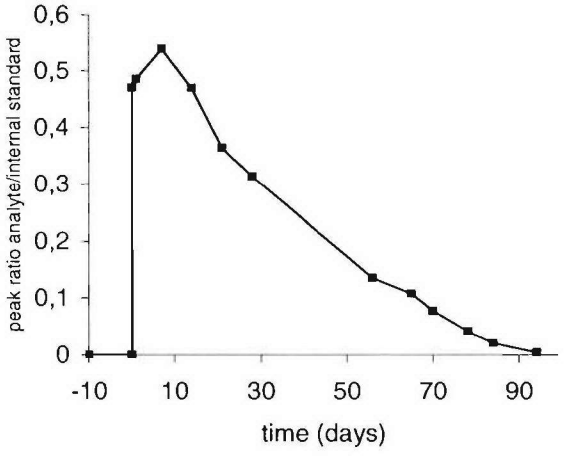


Figure 16 Persistence of sulfur mustard adduct to N-terminal valine residue of hemoglobin in blood of a marmoset after sulfur mustard administration (4.1 mg/kg, i.v.) at t = 0. At the time points indicated blood samples were collected, globin was isolated and analyzed by using the modified Edman degradation for determination of the N-terminal valine adduct. Globin from human blood exposed to d_8-sulfur mustard (10 µM) was used as an internal standard.

The histidine adduct is the most abundant adduct formed after exposure of hemoglobin to sulfur mustard, and is stable upon hydrolysis of globin in 6 N HCl. However, its GC-MS analysis is hampered by the high polarity and poor thermal stability of volatile histidine derivatives. A sensitive method was developed using LC/electrospray tandem MS after derivatization to the N-9-fluorenylmethoxycarbonyl derivative (Noort et al., 1997; Black et al., 1997). Globin was hydrolysed in 6N HCl (110°C, 24 h), the analyte concentrated by extraction on a PRS cation exchange cartridge and, after concentration to dryness, derivatised with 9-fluorenylmethoxycarbonyl chloride. This procedure is rather laborious and proved to be less sensitive than the modified Edman degradation. Recently, MALDI-TOF/MS of intact adducted hemoglobin was explored as a diagnostic tool for the confirmation of exposure to sulfur mustard (Price et al., 2002).

Multiple alkylated species were observed from incubates of hemoglobin with sulfur mustard; however, the methodology has not yet been reported for diagnostic purposes.

3.3.2.3 Application to Human Casualties

Using the modified Edman procedure, the N-terminal valine adduct has been detected in several cases of human exposure to sulfur mustard. The long lifetime of hemoglobin adducts was demonstrated in the case of blood samples from two Iranian CW casualties taken 22-26 days after alleged exposure (Benschop et al., 1997). One victim suffered from skin injuries compatible with sulfur mustard intoxication but had no other injuries; the symptoms of the other victim were only vaguely compatible with sulfur mustard intoxication. The N-terminal valine adduct levels corresponded with those found in human blood after treatment *in vitro* with approximately 0.9 μM sulfur mustard.
The results were confirmed by immunochemical analysis of DNA adducts in lymphocytes from the same blood samples (*vide supra*). Positive results were also reported for blood samples from four Iranian casualties, collected between 5 and 10 days following the alleged CW attack (Black et al., 1997). Concentrations were low, equivalent to 0.3-0.8 ng/ml of the valine adduct. A similar low concentration was found in the blood of a casualty accidentally exposed to sulfur mustard (blood collected 2-3 days after the exposure). Alkylated histidine was detected in blood from the same set of four Iranian casualties, and the accidental casualty. Concentrations were equivalent to 0.7-2.5 ng/ml (Black et al., 1997). It should be noted that in all of the examples above, the concentrations of adduct detected were low in relation to the limits of detection and scrupulous controls must be run during the analyses.

3.3.3 Sulfur Mustard Adducts with Albumin

3.3.3.1 Site of Reaction

Sulfur mustard was shown by Noort et al (1999) to alkylate the cysteine-34 residue in human serum albumin. The site of alkylation was identified in a tryptic digest of albumin from blood exposed to [^{14}C] sulfur mustard. The cysteine-34 residue is the only free cysteine residue in human serum albumin and has a relatively low pKa, caused by intramolecular stabilization of the thiolate anion. It has previously been identified as a nucleophilic site capable of reacting with various electrophiles (Bechtold et al, 1992; Waidyanatha et al., 1998).

3.3.3.2 Analytical Method

A sensitive method for analysis of the sulfur mustard adduct to this residue was developed based on Pronase digestion of alkylated albumin to the tripeptide S-[2-[(hydroxyethyl)thio]ethyl-Cys-Pro-Phe, and detection using micro-LC/tandem MS (Noort et al., 1999; 2004; see Figure 17). Albumin was isolated from blood, digested with Pronase, and the alkylated tripeptide concentrated by extraction on a C18 cartridge. The detection limit for *in vitro* exposure of human blood to sulfur mustard was determined to be 1 nM (see Figure 18), *i.e.*, two orders of magnitude more sensitive than the modified Edman degradation. An additional advantage of this assay is that albumin can be isolated very rapidly from plasma by means of affinity chromatography (Noort et al., 2003; 2004). Compared to the assay for analysis of N-terminal valine adduct (*vide supra*), it can be expected that this assay is less retrospective, due to the faster elimination rate of albumin adducts (half-life of albumin of 20-25 days vs. the life span of hemoglobin of 120 days).

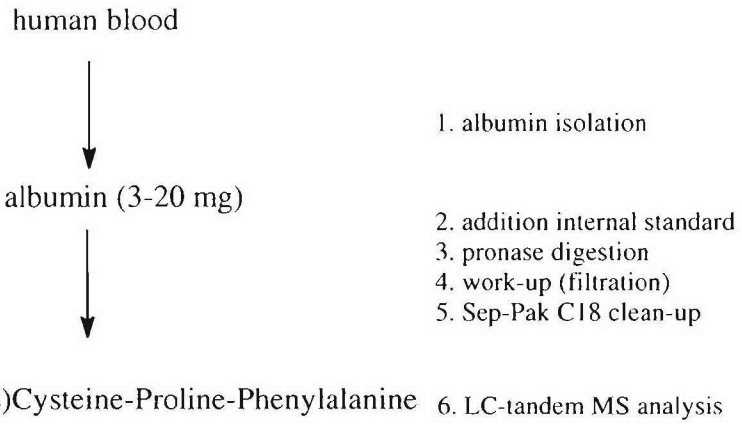


Figure 17 Procedure for analysis of sulfur mustard adduct to cysteine-34 residue in human serum albumin.

Recently, it was shown that various nitrogen mustard-based cytostatics, *e.g.*, melphalan and cyclophosphamide, reacted with the cysteine 34 residue of human serum albumin in an analogous way. The tripeptide-assay could be applied to samples of cancer patients treated with these cytostatics (Noort et al., 2002), which holds promise for optimization of chemotherapy with these agents by intensive screening of adduct levels in patients.

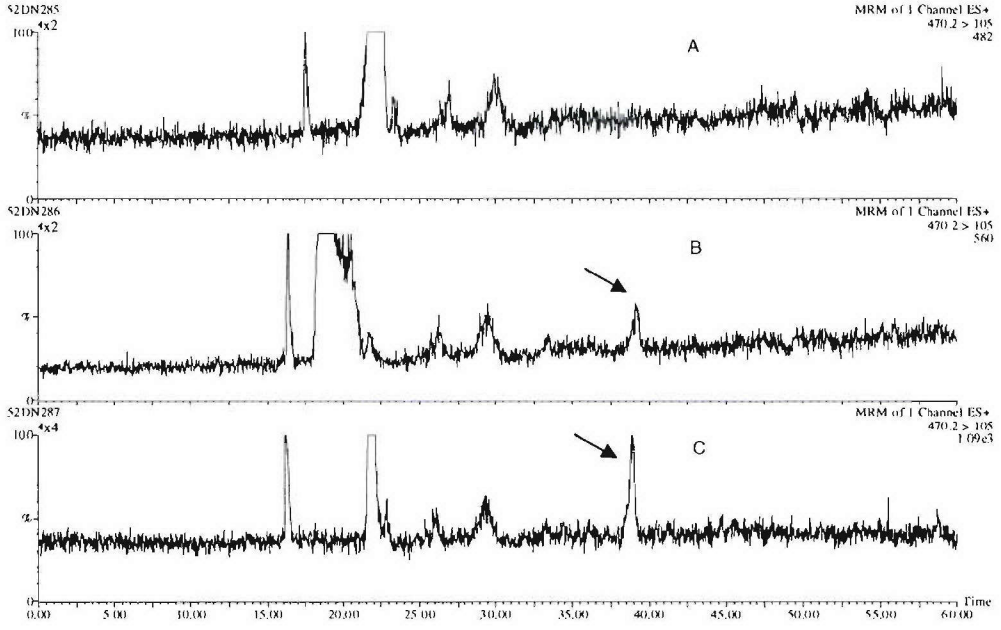


Figure 18 Trace level LC/electrospray tandem MS analysis of (S-HETE)Cys-Pro-Phe in pronase digest of albumin (20 mg) after purification on Sep-Pak C18, measuring the transition m/z 470 (MH+) ---> 105. Albumin was isolated from non-exposed blood (A) or from human blood that was exposed to 1 nM (B). Panel C represents the 1 nM digest after spiking with synthetic (S-HETE)Cys-Pro-Phe. The arrow indicates the peak for (S-HETE)Cys-Pro-Phe.

3.3.3.3 Application to Human Casualties

The analytical procedure for S-[2-[(hydroxyethyl)thio]ethyl-Cys-Pro-Phe was successfully applied to blood samples from nine Iranian casualties of the Iraq-Iran war, all exhibiting skin injuries compatible with exposure to sulfur mustard. The blood samples were collected 8-9 days after the alleged exposure and stored at -70°C.

The albumin adduct was detected in all cases, at levels estimated as corresponding to those after *in vitro* exposure of human blood to mustard concentrations ranging from 0.4 - 1.8 µM.

3.3.4 *Adducts with Aspartic and Glutamic Acid Residues*

3.3.4.1 *Sites of Reaction*

Korte et al. (2002) and Capacio et al. (2004) have recently reported an alternative approach that determines thiodiglycol released by hydrolysis from blood proteins. Both globin and albumin contain numerous aspartic and glutamic acid residues, some of which have been shown to be esterified by sulfur mustard (Black et al., 1997).

3.3.4.2 *Analytical Method*

Blood proteins were precipitated and esterified aspartic and glutamic acid residues hydrolysed with sodium hydroxide (1M NaOH, 2 h, 70°C). After neutralisation, thiodiglycol was extracted from the aqueous layer with ethyl or propyl acetate and analysed as its bis(pentafluorobenzoyl) derivative by GC-MS with negative ion chemical ionization (as described for thiodiglycol in urine or blood in Part 1). The derivative was partly cleaned up by passage through a silica cartridge. The method is very sensitive and could detect thiodiglycol released from swine blood after *in vitro* exposure to 2 nM sulfur mustard, and from monkey blood at least up to 45 days following an intravenous dose of 1 mg/kg. Only a small amount of background interference was observed, equivalent to 0.5 pg thiodiglycol per mg protein.

3.3.5 *Adducts with Keratin*

3.3.5.1 *Sites of Reaction*

The use of sulfur mustard as a vesicant CW agent implies that proteins of the skin are a primary target. It was found that upon exposure of human callus to [^{14}C]sulfur mustard, a significant part of the radioactivity was covalently bound to keratin (Van der Schans et al., 2003). Most of the radioactivity (80%) bound to keratin could be removed by treatment with alkali, indicating the presence of adducts to glutamic and/or aspartic acid residues.

3.3.5.2 *Analytical Method*

A direct detection method was recently developed for these adducts in stratum corneum of human skin based on immunofluorescence microscopy (Van der Schans et al., 2003). Three partial sequences of keratins containing glutamine or asparagine, adducted with a 2-hydroxyethylthioethyl group at the omega-amide function, were synthesized and used as antigens for raising antibodies. After immunization, monoclonal antibodies were obtained with affinity for keratin isolated from human callus exposed to 50 µM sulfur mustard (see Figure 19). In contrast to the immunochemical method for analysis of DNA-sulfur mustard adducts, which involves laborious work-up procedures, this approach opens the way for development of a rapid detection kit that can be applied directly to the skin.

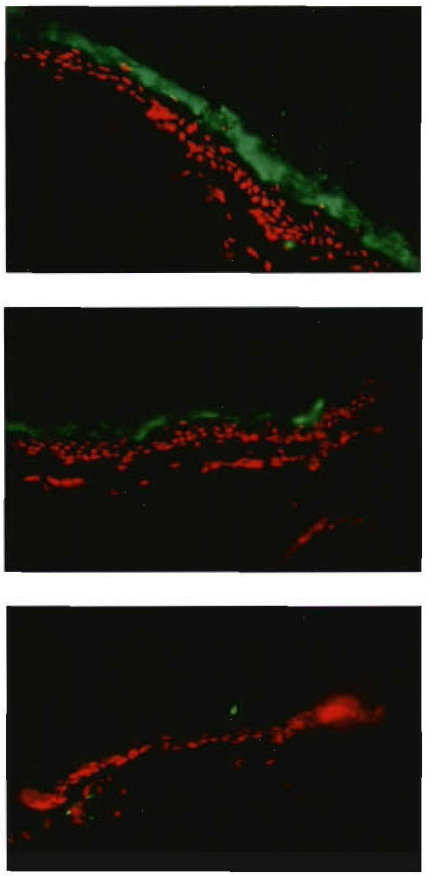


Figure 19 Immunofluorescence microscopy of a cross-section of human skin exposed to saturated sulfur mustard vapor (1 min at 27 °C; Ct ≈ 1040 mg.min.m-3; upper panel) or sulfur mustard (100 μM, 30 min at 27 °C; middle panel) and of unexposed skin (lower panel), using monoclonal antibody 1H10, directed against sulfur mustard adducts to human keratin, in a 1/50 dilution. The photographs are composed from an image obtained for FITC fluorescence (mainly emanating from the stratum corneum; green) and from an image obtained for propidium iodide fluorescence representing DNA (red) in the same cross-section.

3.4 Lewisite

Lewisite is the most important of the organo-arsenical CW agents. Exposure to Lewisite is quite painful, and onset of symptoms occurs rapidly (seconds to minutes) (Goldman et al., 1989), in contrast to sulfur mustard for which a latency period occurs of several hours between exposure and symptoms (Papirmeister et al., 1991). Although it is not known to have been used as a CW agent, Lewisite is still considered a potential threat due to the relative ease of production and its rapid onset of action. Moreover, substantial stockpiles of Lewisite are present in the U.S.A., Russia, and in China abandoned by the Japanese Imperial Army. This may constitute a potential hazard for public health (Watson and Griffin, 1992). The toxicity of Lewisite is *inter alia* caused by the high affinity for the vicinal di-thiol system present in dihydrolipoic acid, a component of the pyruvate dehydrogenase complex, as is the case for other arsenicals (Aposhian, 1989). This prevents the formation of acetyl coenzyme A from pyruvate. The most generally applied method for determination of an arsenical is by atomic absorption spectrometry (AAS) after reduction of the compound to AsH_3. However, this only provides an indication of the presence of the element As against a natural background. Lewisite rapidly hydrolyzes to 2-chlorovinylarsonous acid (CVAA; see

Figure 20) in an aqueous environment such as blood plasma, and analytical methods have focused mainly on the determination of CVAA (see Part 1).

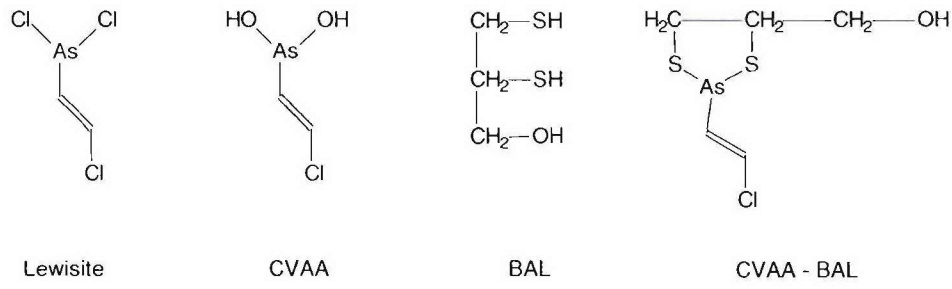


Figure 20 Chemical structures of 2-chlorovinyldichloroarsine (Lewisite; L1), 2-chlorovinylarsonous acid (CVAA), British Anti-Lewisite (BAL) and the CVAA-BAL complex.

3.4.1 Hemoglobin Adduct

3.4.1.1 Site of Reaction

In view of the high affinity of arsenic for thiol functions, it can be expected that Lewisite and CVAA will bind to cysteine residues of proteins. When human blood was incubated with 20 nM to 0.2 mM of [^{14}C]Lewisite, 25-50% of the dose became associated with globin (Fidder et al., 2000). Electrospray tandem MS provided evidence for the presence of a CVAA-crosslink between the cysteine-93 and cysteine-112 residues in β-globin. Whether this was the only type of adduct has not yet been completely elucidated. It must be remarked, however, that this result was in contrast with results obtained by others for the analogous phenyldichloroarsine, for which binding to human hemoglobin could not be observed.

3.4.1.2 Analytical Method

CVAA could be readily displaced from globin (or whole blood) as the CVAA-BAL derivative by addition of 2, 3-dimercaptopropanol (British Anti-Lewisite, BAL) (Figure 20). The latter forms a thermodynamically favored dithiaarsenoline derivative at ambient temperature that can be extracted onto a C18 cartridge. This derivative could be analyzed very sensitively by GC-MS under electron impact conditions, after additional derivatization with heptafluorobutyrylimidazole. The lowest detectable concentration of Lewisite for *in vitro* exposure of human blood was determined to be 1 nM. *In vivo* experiments were performed with guinea pigs (0.25 mg/kg; s.c.). The amount of CVAA-BAL isolated from blood samples clearly decreased with increasing time after exposure, as should be expected. In the blood sample taken 10 days after exposure, the amount of isolated CVAA-BAL had decreased to 10% of the amount at one day after exposure (Fidder et al., 2000).

3.4.1.3 Application to Human Casualties

No examples of biomedical sample analysis following human exposure to Lewisite have been reported.

3.5 Nerve agents

Nerve agents are the most potent of the weaponised CW agents, causing lethality at doses in the µg/kg range. Their use in the last two decades, in the Iraq-Iran conflict,

against Kurdish communities in Iraq, and by terrorists in Japan, has stimulated the development of a number of forensic methods for confirmation of exposure.

3.5.1 Adducts with Acetyl- and Butyrylcholinesterase

3.5.1.1 Site of Reaction

The extremely high toxicity of nerve agents (for a review see Black and Harrison, 1996) can be attributed to the excessive cholinergic stimulation caused by inhibition of acetylcholinesterase (AChE) at neuromuscular junctions and in the central nervous system (CNS). Nerve agents react rapidly with a serine hydroxyl group in the active site of AChE, with the formation of a phosphate or phosphonate ester. The phosphylated enzyme regenerates extremely slowly, rendering the enzyme inaccessible for its parent substrate acetylcholine. In the case of G-agents the intact agent is present in the organism for only several hours. Therefore, intact agents are not considered good targets for retrospective detection of exposure. Interestingly, the chirality around the phosphorus atom has large implications for the toxicity of these agents. The P(-) isomers of G-agents bind to AChE much more rapidly than the P(+) isomers, which are rapidly hydrolyzed by phosphorylphosphatases. Consequently, the P(-) isomers are much more toxic than the P(+) isomers (Benschop and De Jong, 1991). In addition to binding to AChE, sequestration with the closely related plasma protein butyrylcholinesterase (BuChE) and to carboxylesterase (CaE) occurs. After binding to AChE or BuChE, some G-agents undergo a rapid secondary reaction in which one of the substituents of the phosphyl moiety is split off. This process is called ageing. Thus, in the case of inhibition by soman, the PO-pinacolyl bond is cleaved within minutes (see Ordentlich *et al.*, 1991, for a detailed study), and with tabun, the P-N bond is cleaved (Barak et al., 2000; Elhanany et al., 2001). In both cases a negatively charged phosphyl moiety results, which is resistant to reactivation by nucleophiles such as therapeutically applied oximes.

3.5.1.2 Analytical Methods

a) Determination of inhibited enzyme

The oldest method to establish exposure to nerve agents is measurement of the decrease in AChE activity in blood (see Nigg and Knaak (2000) for a review on biomonitoring of organophosphate exposure). The original colorimetric Ellman procedure (Elmann et al., 1961) or modified variations thereof (Worek et al, 1999) is generally used for occupational health screening and therapeutic monitoring of pesticide-poisoned patients. Although the method is rapid and applicable under field conditions, it suffers from serious drawbacks. Firstly, it does not identify the organophosphate. Secondly, the specificity of the method is low, *i.e.*, various unrelated chemicals (*e.g.*, carbamate pesticides) can contribute to inhibition of AChE. Thirdly, it does not provide reliable evidence for organophosphate exposure at inhibition levels less than 20%, which is due to both substantial intra-individual and inter-individual variations, while control activity levels are often not available (Lotti et al., 1995). Finally, it is less suitable for retrospective detection of exposure due to *de novo* synthesis of enzyme. Nevertheless, measurement of AChE inhibition is still the most widely used method for assessment of exposure to nerve agents.

b) Fluoride reactivation of phosphylated binding sites

In principle, organophosphate-inhibited BuChE in human plasma is a persistent (half-life 5-16 days) and relatively abundant (plasma concentration approximately 80 nM) source for biomonitoring of exposure to organophosphate anticholinesterases. Polhuijs

et al. (1997) developed a procedure for the analysis of phosphylated binding sites, *e.g.*, BuChE in plasma or serum, based on reactivation of the phosphylated enzyme with fluoride ions (2M KF, 2 min, 25°C) (see Figure 21). This converts the phosphyl moiety quantitatively into the corresponding phosphono- or phosphorofluoridate, which is simply isolated by solid phase extraction on C18 and quantitated by GC-NPD or GC-MS. In this way, both the origin and the extent of the organophosphate poisoning can be determined. Furthermore, based on the minimal concentrations of phosphono- or phosphofluoridate that can be detected in blood, it is calculated that levels ≥ 0.01% inhibited BuChE should be quantifiable, *i.e.*, at inhibition levels that are several orders of magnitude less than those that can be measured on the basis of decreased AChE activity. The method is limited by spontaneous reactivation and ageing (*i.e.*, loss of the alkyl moiety from the phosphyl group, for instance for soman) of the phosphylated enzyme and by the natural life span of the enzyme. Recently, the fluoride reactivation method has been further evaluated for detection of exposure to sarin (Jakubowski et al., 2004) and VX (Jakubowski et al., 2001). In the latter case, the original nerve agent is not regenerated but the analogous phosphonofluoridate. Significant improvement of the fluoride reactivation has been reported by Degenhardt et al. (2004); detection limits of approximately 10 pg/ml plasma were achieved for various nerve agents. In case of GB this corresponded to 0.09% inhibition on a sample with normal BuChE levels.
In various animal experiments the method has been verified. Figure 22 shows the amount of sarin that could be regenerated from plasma of rhesus monkeys that were injected with a sign free dose of sarin, adjusted to obtain 30% BuChE inhibition. Sarin could be regenerated up to 55 days after injection of the agent.

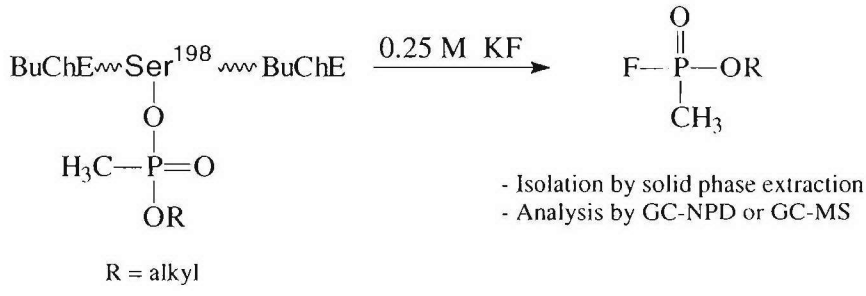
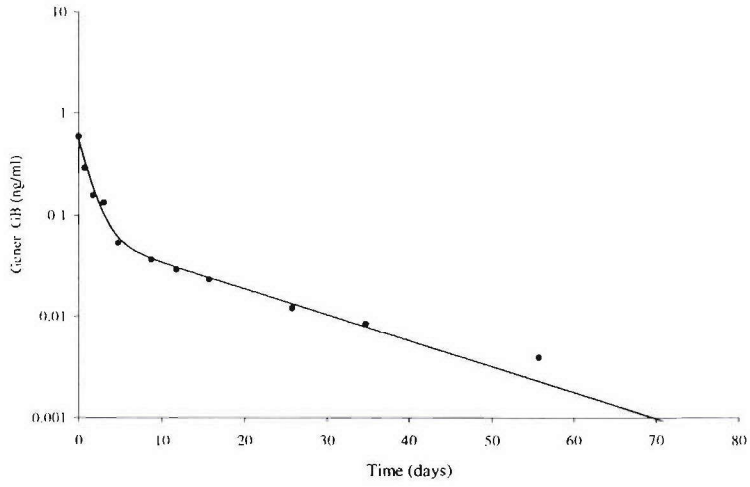


Figure 21 Fluoride reactivation of organophosphate-inhibited butyrylcholinesterase.

c) Hydrolytic displacement of inhibitor from the binding site
An alternative displacement method, reported by Nagao and co-workers (1997, 1998), is based on a lengthy and more complex procedure. Sarin-bound acetylcholinesterase was solubilized from erythrocyte membranes, digested with trypsin (37°C, 24 h), and the hydrolysis product isopropyl methylphosphonic acid released by digestion with alkaline phosphatase (37°C, 48 h). High molecular mass materials were removed by ultrafiltration and the aqueous solution concentrated to dryness. Isopropyl methylphosphonic acid was analysed as its trimethylsilyl derivative by GC-MS.
Figure 22. Fluoride-induced regeneration of sarin from sarin-inhibited BuChE in plasma of a rhesus monkey after administration (i.v.) of GB. The rhesus monkey received a final dose of 0.7 µg/kg.

d) Mass spectrometric determination of cholinesterase adducts



Recently, Noort and co-workers (2002) developed a versatile procedure that is based on straightforward isolation of adducted BuChE from plasma by means of affinity chromatography with a procainamide column, followed by pepsin digestion and LC/electrospray tandem MS analysis of a specific nonapeptide, containing the phosphonylated active site serine-198 residue (see Figure 23). This method surpasses the limitations of the fluoride-reactivation method, since it can also deal with dealkylated (aged) phosphonylated BuChE. Furthermore, the same method could be applied for detection of ChE modifications induced by other inhibitors, *e.g.*, diethyl paraoxon and pyridostigmine bromide, illustrating the broad scope of this approach. This new methodology will also allow the biomonitoring of exposure to several OP pesticides and carbamates in one individual, which is highly relevant within the context of the Food Quality Protection Act of 1996. The latter requires the United States Environmental Protection Agency (EPA) to perform a combined risk assessment for chemicals that produce adverse effects by a common mechanism of toxicity (see, for further reading: http://www.epa.gov/opppsps1/fqpa/). Within the framework of kinetic and mechanistic studies, comprising the interactions of organophosphates with acetyl- and butyrylcholinesterases, Doorn *et al.* (2000, 2001) and Elhanany *et al.* (2001) followed a similar approach by analyzing (modified) peptide fragments in trypsin digests of the cholinesterases with MALDI-TOF/MS.

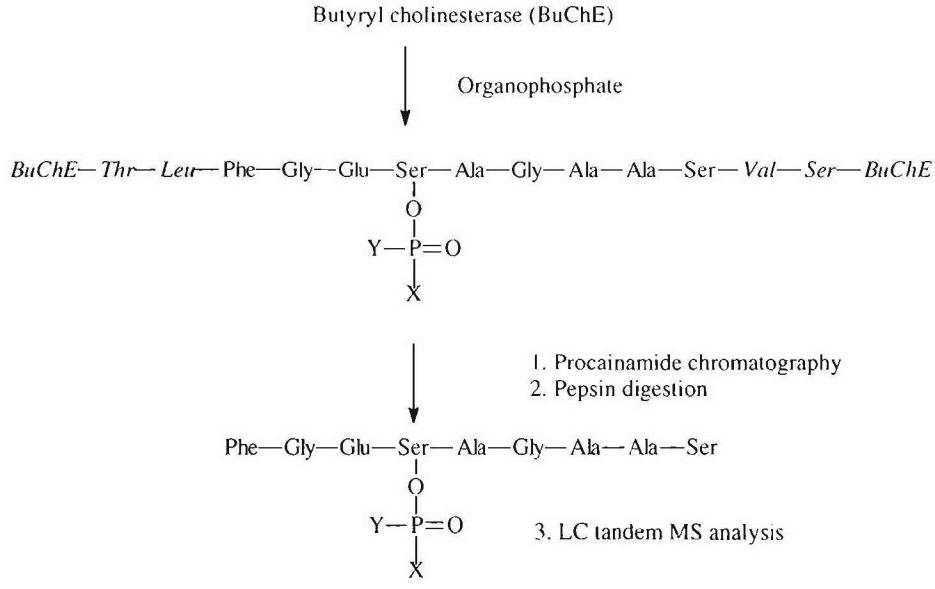


Figure 23 Procedure for LC-tandem MS analysis of phosphylated human butyrylcholinesterase.

3.5.1.3 Application to Human Casualties

All three of the mass spectrometric methods described above were applied to casualties of terrorist attacks in Japan. It should be noted that in most cases the samples were collected within 24 h of the exposure, in contrast to cases of CW exposure to sulfur mustard.

Application of the fluoride reactivation method to serum samples of victims from the Tokyo subway attack, and of the Matsumoto incident, yielded, sarin concentrations in the range of 0.2-4.1 ng/ml serum (Polhuijs et al., 1997). Evidently, these casualties had been exposed to an organophosphate with the formula $iPrO(CH_3)P(O)X$, presumably with X = F (sarin).

The hydrolytic displacement method was applied to four victims killed in the Tokyo subway attack (two died immediately and two later in hospital). Isopropyl methylphosphonic acid was identified at levels sufficient for full scan mass spectra to be obtained using a benchtop quadrupole mass spectrometer (Nagao et al., 1997). Methylphosphonic acid was also identified. Some two years later, methylphosphonic acid was detected in formalin-fixed brain tissues from these victims using a similar procedure (Matsuda et al., 1998).

Finally, mass spectrometric determination of the phosphonylated peptic nonapeptide from butyrylcholinesterase allowed the positive identification of sarin-inhibited enzyme in serum samples from several Japanese victims of the Tokyo subway attack (see Figure 24).

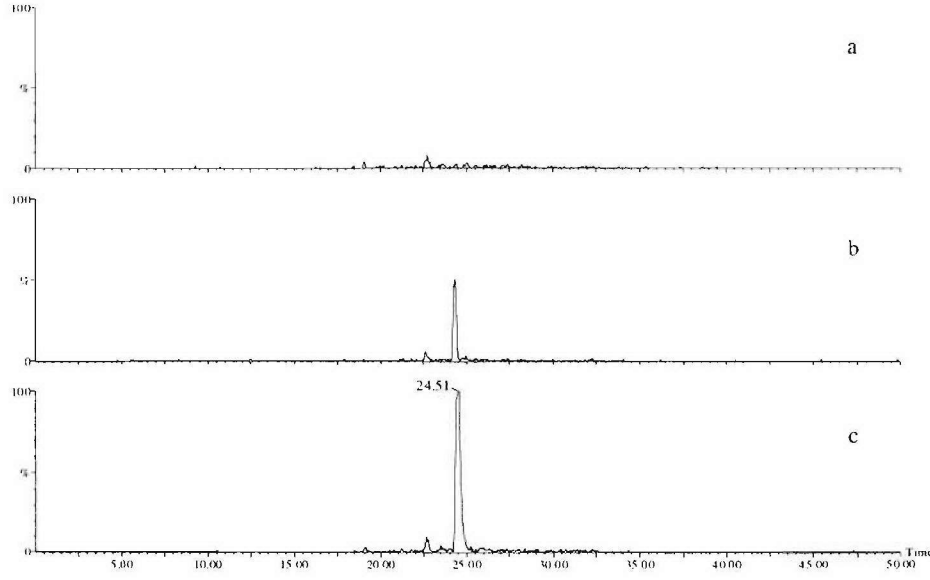


Figure 24 Ion chromatograms of fragment ion m/z 778.4 originating from FGE(S-IMPA)AGAAS, with S-IMPA representing the serine-198 residue conjugated to O-isopropyl methylphosphonic acid, in a pepsin digest of human butyrylcholinesterase (HuBuChE). HuBuChE was isolated from serum of non-exposed human blood (a) and from a Japanese victim of the terroristic attack with sarin in the Tokyo metro (b). Trace (c) represents the pepsin digest shown in trace (a), after spiking with synthetic FGE(S-IMPA)AGAAS.

3.5.2 Adduct with a Tyrosine Residue

3.5.2.1 Site of Reaction

Binding of sarin and soman to a tyrosine residue present in blood has been observed by Black *et al.* (1999). When sarin or soman was incubated with human plasma, phosphonylated tyrosine was observed by LC-MS after Pronase digestion, in addition to phosphonylated serine. The precise site of this residue has not yet been confirmed but it is associated with the albumin fraction. A phosphonylated tryptic peptide [iPrO(CH_3)P(O)]-Tyr-Thr-Lys, consistent with albumin, has been identified but this sequence is also present in other proteins. Before the advent of modern mass spectrometry, diisopropyl fluorophosphate was reported to bind to a tyrosine residue in bovine serum albumin (Murachi, 1963). The adduct with soman is formed less abundantly *in vitro* than that for sarin but, in contrast to the adduct with cholinesterase, there is no indication of ageing occurring.

3.5.2.2 Analytical Method

After Pronase digestion of plasma, the analytes were concentrated on a C18 or C8 cartridge and analysed by LC/electrospray-tandem MS. The adducts have been detected in the blood of guinea pigs 24 h after being exposed to 0.5 LD_{50} doses of sarin and soman. It is not known if they are formed in cases of human exposure.

3.6 Phosgene

The pulmonary agent phosgene was used extensively as a chemical weapon in WW I. Nowadays, it is an important intermediate for industrial production of insecticides, isocyanates, plastics, aniline dyes and resins, with an estimated yearly production of

almost 1 billion pounds. Reliable diagnosis of exposure to phosgene, other than observation of the developing edema by means of chest roentgenology, is not available.

3.6.1 Albumin and Hemoglobin Adducts

3.6.1.1 Sites of Reaction

Noort et al (2000) demonstrated that phosgene binds effectively to albumin and haemoglobin upon in vitro exposure of human blood to [^{14}C]phosgene. Upon Pronase digestion of globin, one of the adducts identified was the pentapeptide O=C-(Val-Leu)-Ser-Phe-Ala, representing amino acids 1-5 of α-globin, with a hydantoin function between the N-terminal valine and leucine. This adduct did not appear suitable as a biomarker of exposure because a peptide with similar properties was detected at trace levels in control samples, possibly formed by reaction of α-globin with carbon dioxide. *Inter alia*, phosgene appears to crosslink the lysine residues 195 and 199 in human serum albumin (see Figures 25 and 26).

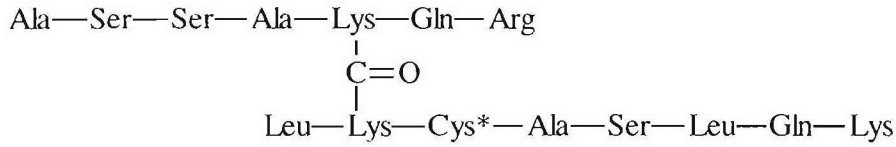


Figure 25 Chemical structure of adduct fragment O=C-(T25-T28) obtained after trypsin digestion of human serum albumin modified at Lys-195 and Lys-199 residues by phosgene.

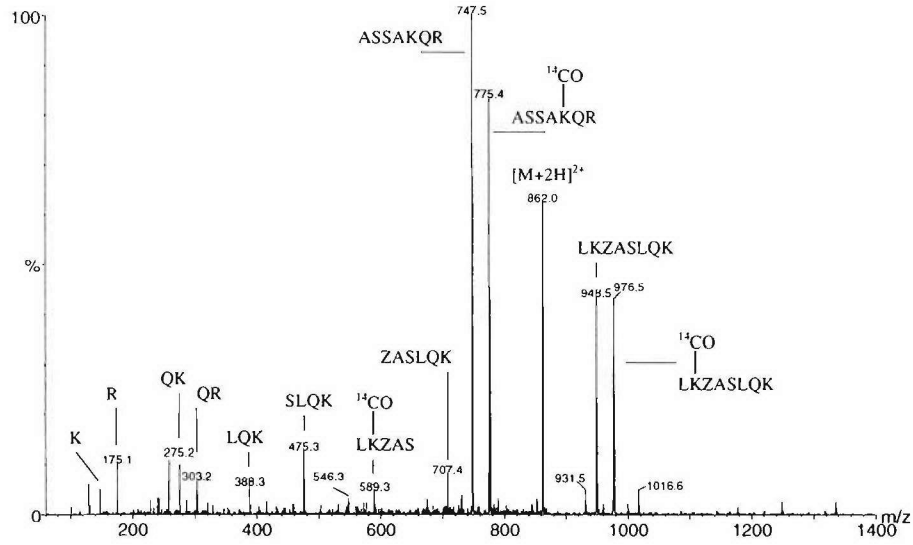


Figure 26 Product ion spectrum of molecular ion [M + 2H]$^{2+}$ (*m/z* 862.0) of O=^{14}C-(T25-T28) in a tryptic digest of albumin isolated from human blood that was exposed to [^{14}C]phosgene.

3.6.1.2 Analytical Method

A method using micro-LC/tandem MS was developed for analysis of the tryptic digest containing the intramolecular albumin lysine-lysine adduct, which enabled the detection of exposure of human blood to ≥ 1 μM phosgene in vitro. The method has not yet been applied to animal or human samples.

3.6.2 Adducts to other proteins

It has been clearly established that phosgene, as the major metabolite of chloroform, is responsible for the acute toxicity of chloroform (Pohl et al., 1977). As part of a study towards the mechanism of the potential carcinogenicity of chloroform, Fabrizi et al (2003) showed that phosgene was able to form adducts with the N-terminus of human histone H2B, through lysine residues.

4 Conclusions

Free metabolites that can be used as unequivocal biological markers of human exposure have been identified for sulphur mustard and nerve agents, and sensitive analytical methods developed for their detection. These have been validated by the analysis of samples from casualties of poisoning by sulphur mustard, sarin and VX. Putative metabolites have been identified in animal studies for nitrogen mustards and Lewisite. Phosgene and cyanide have received little attention with regard to retrospective identification of exposure and significant background levels of metabolites in control subjects may be a complicating factor.

Substantial progress has been made in this research area over the past two decades. New metabolites continue to be identified and new analytical methods developed. Many of the analytical methods have been developed primarily for retrospective identification of exposure in cases of allegations of CW use. In this scenario, low limits of detection are much more important than high throughput. With the current concern for terrorist use of CW agents, new methods of analysis are being developed with greater emphasis on high throughput screening of casualties rather than low or sub-ppb limits of detection. New generations of mass spectrometers will undoubtedly lead to lower limits of detection, particularly in LC-tandem MS. However, a major challenge is the development of sensitive methods that can be performed on the more routine instruments found in most analytical laboratories rather than the very expensive instruments used for research. Another factor that requires consideration is the availability of analytical standards. The most encouraging aspect is that, in combination with the analysis of covalent adducts (see Part 2), there is now a high probability of confirming cases of human exposure to CW agents provided that appropriate samples are collected.

In cases when it is possible to sample shortly after an (alleged) exposure, urine samples might be the samples of choice, because sampling is non-invasive. For sulphur mustard, GC-tandem MS or LC-tandem MS of β-lyase metabolites is the method of choice. For nerve agents, analysis of the alkyl methylphosphonic acids can easily be performed. In case sampling can be performed only after several days, measurement of stable adducts is preferred. In case of sulphur mustard, the albumin tripeptide assay is the method of choice, because it is the least laborious and the most sensitive method. In case of nerve agents, fluoride reactivation is preferred. For rapidly aging compounds like soman and tabun, LC tandem MS analysis of peptide adducts derived from butyrylcholinesterase is the alternative.

Adducts with macromolecules, particularly proteins, offer long-lived biological markers of exposure to CWA, possibly up to several months. Gas or liquid chromatography combined with tandem mass spectrometry, are the methods of choice for unequivocal identification of these adducts or metabolites at trace levels. Several of the developed methods have been applied to actual cases of human exposure to CWA, and have been shown to be highly retrospective.

Many of the methods are highly demanding in terms of detection limits and few of the procedures described are at present easily transferable to routine 'benchtop' mass spectrometer systems. With the exception of ChE inhibition measurements and immunoassays, none of the methods are currently suitable for field laboratories.

However, with the rapid ongoing miniaturization of GC-MS and LC-MS equipment, it can be expected that some of the methods will be applicable on-site within a few years. One of the major challenges of researchers in this field is the lowering of detection limits, since exposures to CWA are often limited to single occasions or to very low levels, and experience has shown that samples may become available several weeks or months after the event. In this respect, the tremendous advances in enhancement of the sensitivity and resolution of electrospray mass spectrometry instruments, and several other hybrid configurations, must be recognized.

At the time of the Iraq-Iran conflict, which was the first conflict in modern times where CW was extensively used, no validated procedures were available for biomedical sample analysis. In the following two decades, more than a dozen different methods have been reported for sulfur mustard and nerve agents, although these are currently available in relatively few laboratories. With the rapid progress being made in the identification of new biomarkers of exposure, combined with improvements in instrumentation, it is becoming increasingly likely that significant exposures to a CW agent can be confirmed, provided that appropriate samples are collected.
Another important issue is the use of alternative matrices, like saliva and nasal lavage fluid (NLF). It should be possible to detect albumin adducts in NLF, and for instance, low molecular metabolites in saliva. The advantage is that these samples can be taken in a non-invasive way, and in case of NLF, the nose is an important *port d'entree*.

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6 Signature

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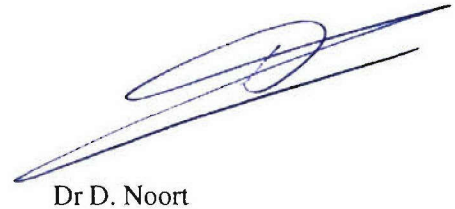

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This report is an update of TNO report PML 2003-A63. In this report an overview is presented of the methods currently available for detection of exposure to a number of chemical warfare agents (CWA), i.e., sulfur mustard, lewisite and nerve agents. Such methods can be applied for various purposes, e.g., diagnosis and dosimetry of exposure of casualties, confirmation of non-exposure, verification of non-adherence to the Chemical Weapons Convention, health surveillance, and forensic purposes. The methods are either based on mass spectrometric analysis of urine or plasma metabolites that result from hydrolysis and/or glutathione conjugation (Part 1), or on mass spectrometric or immunochemical analysis of CWA adducts with DNA or proteins (Part 2). Several of the methods have been successfully applied to actual cases.

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