Chapter 22

MEDICAL DIAGNOSTICS

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INTRODUCTION

In the past, issues associated with chemical warfare agents, including developing and implementing medical countermeasures, field detection, verification of human exposures, triage, and treatment, have primarily been a concern of the military community because most prior experience with chemical warfare agents was limited to the battlefield. However, chemical agents have been increasingly employed against civilian populations, such as in Iraqi attacks against the Kurds and the attacks organized by the Aum Shinrikyo cult in Matsumoto City and the Tokyo subway. The attacks on the World Trade Center in New York City and the Pentagon in Washington, DC, in 2001 have increased concern about the potential large-scale use of chemical warfare agents in a civilian sector. Incidents involving large numbers of civilians have shown that to facilitate appropriate treatment, it is critical to identify not only those exposed, but those who have not been exposed, as well. In addition to health issues associated with exposure, the political and legal ramifications of a chemical warfare attack can be enormous. It is therefore essential that testing for exposure be accurate, sensitive, and rapid.

For the most part, monitoring for the presence of chemical warfare agents in humans, or "biomonitoring," involves examining specimens to determine if an exposure has occurred. Assays that provide definitive evidence of agent exposure commonly target metabolites, such as hydrolysis products and adducts formed following binding to biomolecular entities. Unlike drug efficacy studies in which blood/plasma levels usually focus on the parent compounds, assay techniques for verifying chemical agent exposure rarely target the intact agent because of its limited longevity in vivo. Following exposure, many agents are rapidly converted and appear in the blood as hydrolysis products (resulting from reactions with water) and are excreted in urine. Because of rapid formation and subsequent urinary excretion, the use of these products as markers provides a limited window of opportunity to collect a sample with measurable product. More long-lived markers tend to be those that result from agent interactions with large-molecular–weight targets, such as proteins and DNA. These result from covalent binding of the agent or agent moiety to form macromolecular adducts. As such, the protein acts as a depot for the adducted agent and the residence time is similar to the half-life of the target molecule.

Blood/blood components, urine, and, in rare instances, tissue specimens, termed "sample matrices" or "biomedical samples," can be used to verify chemical agent exposure. However, any sample obtained from an exposed individual may be considered as a potential matrix (eg, blister fluid from sulfur mustard [North Atlantic Treaty Organization (NATO) designation: HD] vesication). Regardless, analyses of these types of samples are inherently difficult because of the matrix's complex composition and the presence of analyte in trace quantities.

Noninvasive urine collection does not require highly trained medical personnel or specialized equipment. Although biomarkers present in urine are usually short-lived (hours to days) metabolites, they can be present in relatively high concentrations in samples obtained shortly after exposure. The collection of blood/plasma should be performed by trained medical personnel. Blood/plasma samples offer potential benefits because both metabolites and the more long-lived adducts of a macromolecular target can be assayed. The effectiveness of using tissue to verify chemical agent exposure is generally limited to postmortem sampling. For example, formalin-fixed brain tissues from fatalities of the Tokyo subway attack were successfully used to verify sarin (NATO designation: GB) as the agent employed in that attack.[1] Other tissue samples can be obtained from the carcasses of animals at the incident site.

Methods that do not directly analyze cholinesterase (ChE) activity typically involve detection systems like mass spectrometry (MS) combined with either gas chromatography (GC) or liquid chromatography (LC) to separate the analyte from other matrix components. MS detection methods are based upon specific and characteristic fragmentation patterns of the parent molecule, making MS detection desirable because it identifies the analyte fairly reliably. Other detection systems, such as nitrogen-phosphorus detection and flame photometric detection, have also been used. Some analytes can be directly assessed, whereas others may require chemical modification (eg, derivatization) to enhance detection or make them more volatile in GC separations. More sophisticated techniques may employ GC or LC with tandem MS (MS-MS) detection systems, allowing more sensitivity and selectivity.

Validating the performance of an analytical technique subsequent to initial in-vitro method development is usually accomplished with in-vivo animal exposure models. Additional information may be gleaned from archived human samples from past exposure incidents. Samples from humans exposed to sulfur mustard during the Iran-Iraq War and a limited number of samples from the Japanese nerve agent attacks have been used to evaluate assay techniques. In the case of some agents, background marker levels are known to exist in nonexposed individuals, making it

difficult to interpret the results of potential incidents. Therefore, in addition to assessing performance in animal models and archived human samples, it is essential to determine potential background levels and incidence of markers in nonexposed human populations. It should be stressed that, for the purpose of exposure verification, results from laboratory testing must be considered along with other information, such as the presentation of symptoms consistent with the agent in question and results from environmental testing.

In the late 1980s the US Army Medical Research Institute of Chemical Defense was tasked by the Department of Defense to develop methods that could confirm potential chemical warfare agent exposure. The US Army Medical Research Institute of Chemical Defense had previously published procedures for verifying exposure to nerve agents and sulfur mustard. These methods primarily focused on GC-MS analysis of hydrolysis products excreted in the urine following exposure to chemical warfare agents. Subsequently, the methods using urine or blood samples were compiled as part of Technical Bulletin Medical 296, titled "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide."[2] The publication was intended to provide clinicians with laboratory tests to detect exposure to chemical warfare agents.

In the mid 1990s, after the publication of Technical Bulletin Medical 296, the military adapted some of the laboratory analytical methods for field-forward use. The concept was demonstrated by the US Army 520th Theater Army Medical Laboratory, which used the Test-Mate OP Kit (EQM Research Inc, Cincinnati, OH) for acetylcholinesterase (AChE) assay and a fly-away GC-MS system. The lengthy preparation of GC and MS samples for analysis in a field environment was one of the reasons that alternative methods of analysis for chemical warfare agents were later examined.

Preexposure treatments or tests to monitor potential chemical agent exposure may be warranted for military personnel and first responders who must enter or operate in chemically contaminated environments. However, laboratory testing may not be as useful for large civilian populations unless there is a clear impending chemical threat. At the same time, determining the health effects of chemical exposure is complex because it can affect the nervous system, respiratory tract, skin, eyes, and mucous membranes, as well as the gastrointestinal, cardiovascular, endocrine, and reproductive systems. Individual susceptibility, preexisting medical conditions, and age may also contribute to the severity of a chemically related illness. Chronic exposures, even at low concentrations, are another concern. In addition to development of diagnostic technologies, strategies to detect chemical agent exposure have become a public health issue.

The transition of laboratory-based analytical techniques to a far-forward field setting can generate valuable information for military or civilian clinicians. In this transition, problems such as data analysis, interpreting complex spectra, and instrument troubleshooting and repair may need addressing. As analytical methods are developed, refined, and sent farther from the laboratory, advanced telecommunication will be needed to provide a direct link between research scientists and field operators; telecommunication will become critical to confirming patient exposure and tracking patient recovery and treatment.

This chapter provides a basic outline and references for state-of-the-art analytical methods presently described in the literature. Methods of verification for exposure to nerve agents, vesicants, pulmonary toxicants, metabolic poisons, incapacitating agents, and riot control agents will be reviewed. Biological sample collection, handling, storage, shipping, and submission will be explained.

NERVE AGENTS

Background

The first organophosphorus (OP) nerve agent, tabun (NATO designation: GA) was developed shortly before and during World War II by German chemist Gerhard Schrader at IG Farbenindustrie in an attempt to develop a commercial insecticide.[3,4,5] Shortly thereafter, sarin was synthesized. Both are extremely toxic. The German government realized the compounds had potential as chemical warfare agents and began producing them and incorporating them into munitions. Subsequently, soman (NATO designation: GD) was synthesized, but only small amounts were produced by the end of the war.[4] Five of the OP compounds are generally regarded as nerve agents: tabun, sarin, soman, cyclosarin (NATO designation: GF), and Russian VX.[4] These compounds demonstrated extreme toxicity, which was attributed to long-lasting binding and inhibition of the enzyme AChE. As a result, the compounds were referred to as "irreversible" inhibitors. Related, but less toxic compounds (ie, "reversible" inhibitors), are becoming widely used therapeutically; for example, in the treatment of Alzheimer's disease. The relative description as reversible or irreversible refers to the length of the binding to the enzyme (Figure 22-1).

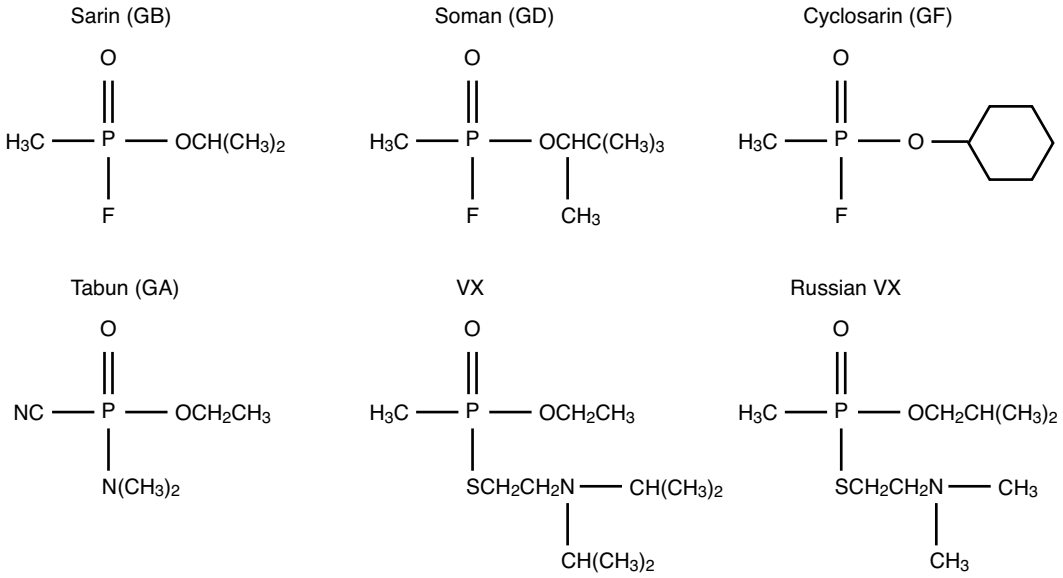


Fig. 22-1. Chemical structures of nerve agents. The nerve agents sarin (GB), soman (GD), and cyclosarin (GF) lose fluorine subsequent to binding to cholinesterase. The agents tabun (GA), VX, and Russian VX lose cyanide and the thiol groups.

Many of the assays developed for exposure verification are based on the interaction of nerve agents with ChE enzymes. Nerve agents inhibit ChE by forming a covalent bond between the phosphorus atom of the agent and the serine residue of the enzyme active site. That interaction results in the displacement or loss of fluorine from sarin, soman, and cyclosarin. The binding of tabun, VX, and Russian VX is different in that the leaving group is cyanide followed by the thiol groups (see Figure 22-1).[6] Spontaneous reactivation of the enzyme or hydrolysis reactions with water can occur to produce corresponding alkyl methylphosphonic acids (MPAs). Alternatively, the loss of the O-alkyl group while bound to the enzyme produces a highly stable organophosphoryl-ChE bond, a process referred to as "aging." Once aging has occurred, the enzyme is considered resistant to reactivation by oximes or other nucleophilic reagents.[4] The spontaneous reactivation and aging rates of the agents vary depending on the O-alkyl group. For example, VX-inhibited red blood cell (RBC) ChE reactivates at an approximate rate of 0.5% to 1% per hour for the first 48 hours, with minimal aging. On the other hand, soman-inhibited ChE does not spontaneously reactivate and has a very rapid aging rate, with a half-time of approximately 2 minutes.[4]

General Clinical Tests

With the exception of ChE analysis, there are no standard clinical assays that specifically test for nerve agent exposure. However, over the years numerous lab-based, non-ChE analytical methods have been developed, and several successfully utilized, to verify nerve agent exposure. For the most part, these employ MS with GC or LC separations. The tests are relatively labor intensive, requiring trained personnel and sophisticated instrumentation not usually available in clinical settings. Most experience using these techniques has come from animal exposure models. These assessments allow for determination of test sensitivity and biomarker longevity in experimental models. In humans, there is limited experience from accidental and terror-related exposures. This chapter will review assays for chemical warfare agent exposure that have been published in the literature and how they have been applied in potential exposure situations

Assay of Parent Compounds

Analyzing for parent nerve agents from biomedical matrices, such as blood or urine, is not a viable diagnostic technique for retrospective detection of exposure.[7] Parent agents are relatively short-lived because of rapid hydrolysis and binding to plasma and tissue proteins, imposing unrealistic time restraints on sample collection. The short residence time is especially profound with the G agents (relative to VX). Following the intravenous administration of soman at 2 times the median lethal dose (LD_{50}) results in parent agent detection at toxicologically relevant levels for 104 and 49 minutes in guinea pigs and marmosets, respectively; rapid elimination was reflected in terminal

half-life rates (16.5 min for guinea pigs; 9 min for marmosets).[8] Inhalation experiments using nose-only exposure of guinea pigs to 0.8 × LCt_{50} (the vapor or aerosol exposure that is lethal to 50% of the exposed population) agent demonstrate terminal half-lives of approximately 36 and 9 minutes for sarin and soman, respectively.[9] In contrast, similar studies with VX in hairless guinea pigs and marmosets indicate VX is more persistent than the G agents.[10] These studies show that VX can be found at acutely toxic levels for 10 to 20 hours following intravenous administration at a dose one or two times the LD_{50}, with terminal elimination rates of 98 minutes (1 times the LD_{50} in hairless guinea pigs), 165 minutes (2 times the LD_{50}), and 111 minutes in marmosets (at a dose equivalent to 1 LD_{50} in hairless guinea pigs). Percutaneous administration of the LD_{50} of VX to hairless guinea pigs demonstrated relatively low blood levels (140 pg/mL), which reached a maximum after approximately 6 hours.[10] Because the route of human exposure to VX would most likely occur percutaneously, the time frame of 6 hours may be the more relevant assessment of its persistence in blood. This allows very limited time for sample collection and analysis. Others have demonstrated that VX can be assayed from spiked rat plasma.[11] These authors noted that 53% of the VX was lost in spiked plasma specimens after 2 hours. The disappearance was attributed to the enzyme action of the OP hydrolase splitting or to cleavage of the sulfur-phosphorus bond to form diisopropyl aminoethanethiol (DAET) and ethyl methylphosphonic acid (EMPA).[11]

Assay of Hydrolysis Compounds

Analytical Methods

An alternative approach to direct assay of parent nerve agents is to measure metabolic or hydrolysis products in specimens. These compounds are produced in vivo as a result of hydrolysis or detachment following spontaneous regeneration of the AChE enzyme. Studies of parent nerve agents with radioisotopically labeled phosphorus (^{32}P) or hydrogen (^{3}H) in animals suggest that agents are rapidly metabolized and hydrolyzed in the blood and appear in the urine as their respective alkyl MPAs.[12–15] This observation led to the development of assays for alkyl MPAs in biological samples,[16] the applicability of which was subsequently demonstrated in animals exposed to nerve agents.[17] The common products found are isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid, cyclohexyl methylphosphonic acid, and EMPA derived from sarin, soman, cyclosarin, and VX, respectively (Figure 22-2). Additionally for VX, hydrolysis of the sulfur-phosphorus bond occurs, yielding DAET and EMPA. The formation and assay of DAET has been reported in rat plasma spiked with VX.[11] Furthermore, the presence of diisopropyl aminoethyl methyl sulfide, presumably resulting from the in-vivo methylation of DAET, has been reported in human exposures.[18] To date, numerous variations of the alkyl MPA assay for biological fluids, such as plasma and urine, have been developed. These include GC separations with MS,[18–20] tandem MS (MS-MS),[18,19,21,22] and flame photometric detection.[23,24] Other methods involving LC with MS-MS[25] and indirect photometric detection[26] have also been reported (Table 22-1).

Application to Human Exposures

The utility of some methodologies has been demonstrated in actual human exposure incidents. Most involve assays of urine and plasma or serum. Tsuchihashi et al[18] demonstrated the presence of EMPA in the serum of an individual assassinated with VX in Osaka, Japan, in 1994. As mentioned earlier, these authors also reported the presence of diisopropyl aminoethyl methyl sulfide, which resulted from the in-vivo methylation of DAET subsequent to cleavage of the sulfur-phosphorus bond. Reported concentrations in serum collected 1 hour after exposure were 143 ng/mL diisopropyl aminoethyl methyl sulfide and 1.25 μg/mL for EMPA.

The Aum Shinrikyo cult attacked citizens twice in Japan using sarin. The first was in an apartment complex in Matsumoto City, where approximately 12 liters of sarin were released using a heater and fan. According to police reports, 600 inhabitants in the surrounding area were harmed, including 7 who were killed. In the second attack, sarin was released into the Tokyo subway, resulting in more than 5,000 casualties and 10 deaths.[27] Assay of hydrolysis products as a definitive marker were used to verify that sarin was the agent employed in these events. Minami et al[23] and Nakajima et al[24] demonstrated the presence of IMPA or MPA in victims' urine following sarin exposure in the Tokyo and Matsumoto attacks, respectively. These methods used GC separations of the prepared urine matrix coupled with flame photometric detection. In the Matsumoto incident, urinary concentrations of IMPA and MPA, as well as the total dose of the sarin exposure, were reported.[24] For one victim, MPA concentrations were 0.14 and 0.02 ug/mL on the first and third days after exposure, and 0.76, 0.08, and 0.01 ug/mL for IMPA, respectively, on the first, third, and seventh days after exposure.[24] In this case, the individual was estimated to have been exposed to 2.79 mg of sarin.

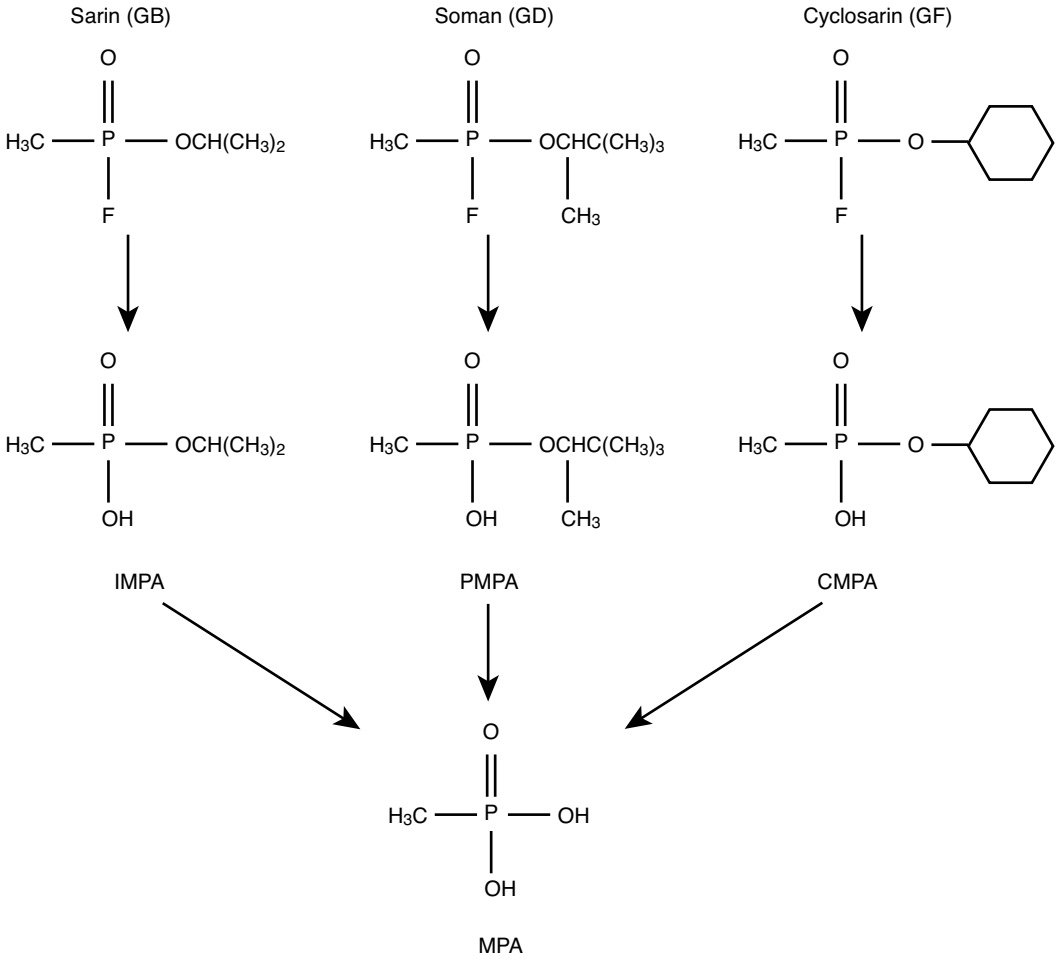


Fig. 22-2. Hydrolysis pathway of sarin (GB), soman (GD), and cyclosarin (GF). Hydrolysis pathway of nerve agents proceeds through the alkyl methylphosphonic acids IMPA, PMPA, and CMPA to MPA. Analysis of the alkyl methylphosphonic acids allows identification of the parent agent, while assay of MPA is nonspecific.
CMPA: cyclohexyl methylphosphonic acid
IMPA: isopropyl methylphosphonic acid
MPA: methylphosphonic acid
PMPA: pinacolyl methylphosphonic acid

Although the report on the Tokyo[23] incident did not directly indicate urinary concentrations, total sarin exposure was estimated. The exposure estimates in a comatose individual was 0.13 to 0.25 mg/person and 0.016 to 0.032 mg/person in a less severely exposed casualty.[23] These numbers are approximately 10-fold less than those reported by Nakajima et al[24] for a severely intoxicated patient. Consistent with rapid elimination, the maximum urinary concentration of these compounds was reported to have occurred in 12 hours of exposure. Using LC-MS-MS methods, Noort et al[25] and Polhuijs et al[28] detected IMPA in serum samples from both the Tokyo and Matsumoto incidents. This assay involves fairly sophisticated instrumentation, but allows for a simplified sample processing procedure. Reported serum concentrations ranged from 2 to 127 ng/mL and 2 to 135 ng/mL in the Tokyo and Matsumoto incidents, respectively.[25,28] Samples were obtained 1.5 hours after the incident. In some cases a second sample was obtained 2 to 2.5 hours after the incident; in those samples, the authors report significantly lower IMPA concentrations consistent with the rapid elimination of these compounds. The sarin dose in both incidences was calculated to be 0.2 to 15 mg/person.[25] These reported values for sarin exposure are in the range of those reported by Nakajima et al[24] and are approximately 10-fold greater than those reported by Minami et al.[23]

Alkyl MPAs provide a convenient marker for determining exposure to nerve agents. Numerous

TABLE 22-1

ANALYTICAL METHODS FOR ASSAY OF NERVE AGENT HYDROLYSIS PRODUCTS*

Sample Matrix	Product Identified	Analytical Method
Blood, Plasma, Urine, Lung Tissue	IMPA, CMPA, PMPA	GC-MS[1,2]
Serum, Urine	EMPA, IMPA, PMPA	GC-MS, GC-MS-MS[3]
Plasma	DAET	GC-MS[4]
Urine	EMPA, IMPA, MPA	GC-FPD[5]
Urine	IMPA, MPA	GC-FPD[6]
Serum	EMPA, DAEMS	GC-MS, GC-MS-MS[7]
Serum, Urine, Saliva	EMPA, IMPA, PMPA	GC-MS[8]
Urine	EMPA, IMPA, CMPA, PMPA, GA acid	GC-MS-MS[9]
Urine	IMPA	LC-MS-MS[10,11]
Serum	EMPA, IMPA, MPA PMPA	Indirect Photometric Detection Ion Chromatography[12]
Urine, Saliva	EMPA, IMPA, CMPA, MPA, PMPA	LC-MS-MS[13]
Urine	EMPA, RVX acid, IMPA, PMPA, CMPA, GA acid, GA diacid	GC-MS-MS[14]

* Although the sample matrices and analytical methods for some of the assays are similar, the authors specifically identified the products listed.

CMPA: cyclohexyl methylphosphonic acid
DAEMS: diisopropyl aminoethyl methyl sulfide (resulting from the metabolic methylation of DAET)
DAET: diisopropyl aminoethanethiol
EMPA: ethyl methylphosphonic acid
FPD: flame photometric detection
GA: tabun
GC: gas chromatography
IMPA: isopropyl methylphosphonic acid
LC: liquid chromatography
MPA: methylphosphonic acid
MS: mass spectrometry
PMPA: pinacolyl methylphosphonic acid
RVX: Russian VX

Data sources: (1) Shih ML, Smith JR, McMonagle JD, Dolzine TW, Gresham VC. Detection of metabolites of toxic alkylmethylphosphonates in biological samples. *Biol Mass Spectrom.* 1991;20:717–723. (2) Shih ML, McMonagle JD, Dolzine TW, Gresham VC. Metabolite pharmacokinetics of soman, sarin, and GF in rats and biological monitoring of exposure to toxic organophosphorus agents. *J Appl Toxicol.* 1994:14:195–199. (3) Fredriksson SA, Hammarström LG, Henriksson L, Lakso HA. Trace determination of alkyl methylphosphonic acids in environmental and biological samples using gas chromatography/negative-ion chemical ionization mass spectrometry and tandem mass spectrometry. *J Mass Spectrom.* 1995;30:1133–1143. (4) Bonierbale E, Debordes L, Coppet L. Application of capillary gas chromatography to the study of hydrolysis of the nerve agent VX in rat plasma. *J Chromatogr B Biomed Sci Appl.* 1997;688:255–264. (5) Minami M, Hui DM, Katsumata M, Inagaki H, Boulet CA. Method for the analysis of methylphosphonic acid metabolites of sarin and its ethanol-substituted analogue in urine as applied to the victims of the Tokyo sarin disaster. *J Chromatogr B Biomed Sci Appl.* 1997;695:237–244. (6) Nakajima T, Sasaki K, Ozawa H, Sekjima Y, Morita H, Fukushima Y, Yanagisawa N. Urinary metabolites of sarin in a patient of the Matsumoto incident. *Arch Toxicol.* 1998;72:601–603. (7) Tsuchihashi H, Katagi M, Nishikawa M, Tatsuno M. Identification of metabolites of nerve agent VX in serum collected from a victim. *J Anal Toxicol.* 1998;22:383–388. (8) Miki A, Katagi M, Tsuchihashi H, Yamashita M. Determination of alkylmethylphosphonic acids, the main metabolites of organophosphorus nerve agents, in biofluids by gas chromatography-mass spectrometry and liquid-liquid-solid-phase-transfer-catalyzed pentafluorobenzylation. *J Anal Toxicol.* 1999;23:86–93. (9) Driskell WJ, Shih M, Needham LL, Barr DB. Quantitation of organophosphorus nerve agent metabolites in human urine using isotope dilution gas chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2002;26:6–10. (10) Noort D, Hulst AG, Platenburg DH, Polhuijs M, Benschop H. Quantitative analysis of O-isopropyl methylphosphonic acid in serum samples of Japanese citizens allegedly exposed to sarin: estimation of internal dosage. *Arch Toxicol.* 1998;72:671–675. (11) Polhuijs M, Langenberg JP, Noort D, Hulst AG, Benschop HP. Retrospective detection of exposure to organophosphates: analyses in blood of human beings and rhesus monkeys. In: Sohns T, Voicu VA, eds. *NBC Risks: Current Capabilities and Future Perspectives for Protection*. Dordrecht, Holland, Netherlands: Kluwer Academic Publishers; 1999:513–521. (12) Katagi M, Nishikawa M, Tatsuno M, Tsuchihashi H. Determination of the main hydrolysis products of organophosphorus nerve agents, methylphosphonic acids, in human serum by indirect photometric detection ion chromatography. *J Chromatogr B Biomed Sci Appl.* 1997;698:81–88. (13) Hayes TL, Kenny DV, Hernon-Kenny L. Feasibility of direct analysis of saliva and urine for phosphonic acids and thiodiglycol-related species associated with exposure to chemical warfare agents using LC-MS/MS. *J Med Chem Def.* 2004;2:1-23. (14) Barr JR, Driskell WJ, Aston LS, Martinez RA. Quantitation of metabolites of the nerve agents sarin, soman, cyclosarin, VX, and Russian VX in human urine using isotope-dilution gas chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2004;28:371–378.

modifications of the assay for these compounds have been developed, and several have been applied to human exposure cases. Important factors to consider when anticipating using this test are the extent of exposure and time elapsed since the event. In most cases, hydrolysis products are not expected to be present for more than 24 to 48 hours following exposure; however, one of the most severely poisoned victims of the Matsumoto sarin attack had measurable IMPA in the urine on the seventh day after the incident. In this particular case, extremely depressed AChE values, in range of 5% to 8% of normal,[24] further indicated the extent of exposure (Table 22-2).

Assay of Adducts to Biomolecules

The relatively rapid excretion and short-lived presence of urinary hydrolysis products imposes time restrictions for collecting a viable sample. Efforts to increase the sampling window have taken advantage of the interactions between chemical warfare agents and biological targets with large molecular weights (adducts to biomolecules), such as proteins. The reaction of chemical agents with large molecules provides a pool of bound compound that can be tested to verify exposure. Theoretically, the longevity of the marker is consistent with the in-vivo half-life of the target molecule, provided that the binding affinity is high enough to prevent spontaneous reactivation. Binding of nerve agents to ChE targets has been one of the primary interactions leveraged in assay development. Several assays have been developed based on variations of this concept.

Analytical Methods

Polhuijs et al[29] developed an assay technique based on observations of earlier findings that sarin-inhibited ChE could be reactivated with fluoride ions.[30–32] The displacement of covalently bound sarin to butyrylcholinesterase (BChE) was accomplished by incubating inhibited plasma with fluoride to form free enzyme

TABLE 22-2

METHODS USED TO CONFIRM HUMAN EXPOSURES TO NERVE AGENTS VIA ASSAY OF HYDROLYSIS PRODUCTS

Agent/Incident	Sample Matrix	Product Identified	Concentration Reported	Analytical Method
GB, Tokyo, Japan	Urine	EMPA, IMPA, MPA	NR	GC-FPD[1]
GB, Matsumoto, Japan	Urine	IMPA MPA	0.76–0 .01 μg/mL 0.14–0.02 μg/mL	GC-FPD[2]
GB, Matsumoto and Tokyo, Japan	Serum	IMPA	Matsumoto (2–135 ng/mL) Tokyo (2–127 ng/mL)	LC-MS-MS[3,4]
VX, Osaka, Japan	Serum	EMPA, diisopropylaminoethyl methyl sulfide	1.25 μg/mL 143 ng/mL	GC-MS, GC-MS-MS[5]

EMPA: ethyl methylphosphonic acid
FPD: flame photometric detection
GB: sarin
GC: gas chromatography
IMPA: isopropyl methylphosphonic acid
LC: liquid chromatography
MPA: methylphosphonic acid
MS: mass spectrometry
NR: not reported

Data sources: (1) Minami M, Hui DM, Katsumata M, Inagaki H, Boulet CA. Method for the analysis of methylphosphonic acid metabolites of sarin and its ethanol-substituted analogue in urine as applied to the victims of the Tokyo sarin disaster. *J Chromatogr B Biomed Sci Appl.* 1997;695:237–244. (2) Nakajima T, Sasaki K, Ozawa H, Sekjima Y, Morita H, Fukushima Y, Yanagisawa N. Urinary metabolites of sarin in a patient of the Matsumoto incident. *Arch Toxicol.* 1998;72:601–603. (3) Noort D, Hulst AG, Platenburg DH, Polhuijs M, Benschop H. Quantitative analysis of O-isopropyl methylphosphonic acid in serum samples of Japanese citizens allegedly exposed to sarin: estimation of internal dosage. *Arch Toxicol.* 1998;72:671–675. (4) Polhuijs M, Langenberg JP, Noort D, Hulst AG, Benschop HP. Retrospective detection of exposure to organophosphates: analyses in blood of human beings and rhesus monkeys. In: Sohns T, Voicu VA, eds. *NBC Risks: Current Capabilities and Future Perspectives for Protection*. Dordrecht, Holland, Netherlands: Kluwer Academic Publishers; 1999:513–521. (5) Tsuchihashi H, Katagi M, Nishikawa M, Tatsuno M. Identification of metabolites of nerve agent VX in serum collected from a victim. *J Anal Toxicol.* 1998;22:383–388.

plus the parent agent (isopropyl methylphosphonofluoridate). Following isolation from the matrix with solid phase extraction techniques, the agent was then analyzed using GC with MS or other appropriate detection systems. Other research has demonstrated conceptually similar approaches for detecting tabun[28] and VX.[33] In the case of tabun, the cyanide group, which is initially lost upon binding to the enzyme, is replaced with fluorine, leading to the formation of O-ethyl N,N-dimethyl-phosphoramidofluoridate, a fluorinated analog of tabun.[28] Similarly, the thiol group in VX, which is initially lost upon binding to the enzyme, is replaced by fluorine, resulting in a fluorinated analog of VX (ethyl methyl-phosphonofluoridate; VX-G).[33] Variations and improvements of the fluoride regeneration procedure have evolved to enhance test sensitivity by optimizing agent extraction, increasing injection volumes (thermal desorption and large volume injector), and using alternate detection formats (eg, flame photometric detection, positive ion chemical ionization, and high-resolution electron impact MS).[6,33] Additionally, Jakubowski et al[34] have successfully applied the procedure to RBCs.

With regard to the ability of a fluoride ion to regenerate soman bound to BChE, it is well known that the process of aging would preclude release from the enzyme. However, studies have indicated that the fluoride ion regeneration process, as applied to soman-poisoned animals, has produced contrary results.[35] These studies suggest that soman can be displaced from sites where aging does not play a significant role. Black et al[36] have demonstrated that both sarin and soman bind to tyrosine residues of human serum albumin. The observation that the alkyl group remained intact, in particular for soman, argues that binding to this site does not result in aging as seen with ChEs.[36] Similarly, carboxylesterase, known to exist in high quantities in rats and mice, has been shown to form adducts with soman.[37–41] Moreover, soman formation has been demonstrated via fluoride-induced regeneration of soman-inhibited carboxylesterase in rat plasma[37] and purified human albumin.[35] Although the presence of carboxylesterase in significant amounts is questionable in humans, the albumin provides a potential source of the protein from which the agent can be regenerated. Currently the utility of fluoride regeneration in human exposures involving soman is unclear. More studies are needed to clarify the utility of fluoride regeneration in humans with soman exposure following confirmed events.

Nagao et al[42,43] employed a different approach, exploiting sarin bound to AChE, using blood as the matrix. This procedure detected IMPA, following its release from the sarin-AChE complex, using an alkaline phosphatase digestion process. The analytical technique used is similar to numerous other GC-MS assays for hydrolysis products.

Another approach based on OP binding to BChE has been reported by Fidder et al.[44] This method involves digesting BChE to produce nonapeptide fragments containing the serine-198 residue to which nerve agents bind. Analyzing nonapeptides employs LC-MS-MS techniques. The utility of this method was demonstrated by analyzing two archived samples from the Tokyo subway terrorist attack. The authors reported results similar to a previous analysis of those samples, in this case using the fluoride regeneration procedure.[44] A reported advantage of this technique is that aged or nonaged OPs can be successfully identified. In the case of sarin-inhibited enzyme, the serine-198 is conjugated to IMPA; for soman following loss of the pinacolyl alkyl group (ie, aging), MPA was found bound to the serine residue. In addition, the procedure was useful for detecting BChE adducted to OP pesticides as well as non-OP anti-ChEs, such as pyridostigmine.[44] A limitation of the assay is that agent identity is needed for MS analysis.[45] For this reason, an extension of this procedure was developed that uses a generic approach.[45] The method employed a chemical modification of the phosphyl group on the serine residue to a common nonapeptide, regardless of the specific agent involved.[45] Because a common nonapeptide is the outcome, a single MS method was employed in the analysis (Table 22-3).[45]

Application to Human Exposures

The fluoride ion regeneration procedure[29] was used to analyze serum from exposed individuals in the Aum Shinrikyo terrorist attacks at Matsumoto and in the Tokyo subway. As previously indicated, this procedure is based upon the use of fluoride ion to regenerate the parent agent and free BChE. The amount of regenerated sarin from serum ranged from 1.8 to 2.7 ng/mL in the Matsumoto incident and 0.2 to 4.1 ng/mL in the Tokyo attacks.[29]

Although unable to detect MPA or IMPA directly from the blood of victims of the Tokyo subway attack, Nagao et al[42,43] detected these compounds after alkaline phosphatase digestion of the sarin-AChE complex. However, the authors did not report MPA or IMPA concentrations. Although not directly relevant to diagnostic testing, a conceptually similar approach was also applied to formalin-fixed brain tissues (GB-bound AChE) from victims of the Tokyo subway attack.[1] The assays conducted on frozen cerebral cortex did not detect MPA or IMPA. Similar studies with formalin-fixed cerebellum tissue resulted in detecting only MPA. The

TABLE 22-3

ANALYTICAL METHODS USING ADDUCTS TO BIOMOLECULES

Sample Matrix	Product Identified	Analytical Method
Plasma/serum	GA, GB	GC-NPD[1,2]
Red blood cell	IMPA, MPA	GC-MS[3,4]
Brain (cerebellum)	MPA	GC-MS[5]
Plasma/serum	VX-G	GC-FPD/GC-MS[6]
Plasma/serum	Phosphylated nonapeptides from BChE	LC-MS-MS[7]
Plasma/serum	GA, GB, GF, VX-G	GC-MS/GC-MS(HR)[8]
Plasma/serum/red blood cell	GB	GC-MS[9]
Plasma/serum	Phosphylated nonapeptides from BChE- derivatized	LC-MS-MS[10]

BChE: butyrylcholinesterase
FPD: flame photometric detection
GA: tabun
GB: sarin
GC: gas chromatography
GF: cyclosarin
HR: high resolution
IMPA: isopropyl methylphosphonic acid
LC: liquid chromatography
MPA: methylphosphonic acid
MS: mass spectrometry
NPD: nitrogen-phosphorus detector
VX-G : ethyl methylphosphonofluoridate

Data sources: (1) Polhuijs M, Langenberg JP, Benschop HP. New method for retrospective detection of exposure to organophosphorus anticholinesterases: application to alleged sarin victims of Japanese terrorists. *Toxicol Appl Pharmacol.* 1997;146:156–161. (2) Polhuijs M, Langenberg JP, Noort D, Hulst AG, Benschop HP. Retrospective detection of exposure to organophosphates: analyses in blood of human beings and rhesus monkeys. In: Sohns T, Voicu VA, eds. *NBC Risks: Current Capabilities and Future Perspectives for Protection*. Dordrecht, Holland, Netherlands: Kluwer Academic Publishers; 1999:513–21. (3) Nagao M, Takatori T, Matsuda Y, et al. Detection of sarin hydrolysis products from sarin-like organophosphorus agent-exposed human erythrocytes. *J Chromatogr B Biomed Sci Appl.* 1997;701:9–17. (4) Nagao M, Takatori T, Matsuda Y, Nakajima M, Iwase H, Iwadate K. Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. *Toxicol Appl Pharmacol.* 1997;144:198–203. (5) Matsuda Y, Nagao M, Takatori T, et al. Detection of the sarin hydrolysis product in formalin-fixed brain tissues of victims of the Tokyo subway terrorist attack. *Toxicol Appl Pharmacol.* 1998;150:310–320. (6) Jakubowski EM, Heykamp LS, Durst HD, Thompson SA. Preliminary studies in the formation of ethyl methylphosphonofluoridate from rat and human serum exposed to VX and treated with fluoride ion. *Anal Lett.* 2001;34:727–737. (7) Fidder A, Hulst AG, Noort D, et al. Retrospective detection of exposure to organophosphorus anti-cholinesterases: mass spectrometric analysis of phosphylated human butyrylcholinesterase. *Chem Res Toxicol.* 2002;15:582–590. (8) Degenhardt CE, Pleijsier K, van der Schans MJ, et al. Improvements of the fluoride reactivation method for the verification of nerve agent exposure. *J Anal Toxicol.* 2004;28:364–371. (9) Jakubowski EM, McGuire JM, Evans RA, et al. Quantitation of fluoride ion released sarin in red blood cell samples by gas chromatography-chemical ionization mass spectrometry using isoptope dilution and large-volume injection. *J Anal Toxicol.* 2004;28:357–363. (10) Noort D, Fidder A, van der Schans MJ, Hulst AG. Verification of exposure to organophosphates: generic mass spectrometric method for detection of human butyrylcholinesterase adducts. *Anal Chem.* 2006;78:6640–6644.

inability to detect IMPA was due to hydrolysis during the 2-year storage period. The inability to detect hydrolysis products in the cerebral cortex as opposed to the cerebellum was reportedly consistent with the relative AChE activity detected in each tissue. The study authors state that this is the first verification of nerve agent exposure using formalin-fixed brains.[1]

Due to the limited number of human exposures to nerve agents, it is difficult to fully ascertain the advantages and disadvantages of various definitive testing methodologies. Numerous assays to detect hydrolysis products in blood or urine have been developed; some have been employed in exposure incidents. The disadvantage of these methods stems from the relatively rapid agent elimination and resultant limited opportunity to obtain specimens. The advantage of using adducts formed with large-molecular–weight targets (AChE or BChE) is a longer time frame (relative to that of hydrolysis products) to verify exposures. Some investigations have indicated that methods employing BChE provide benefits over those with AChE because BChE is more abundant in blood.[44] Assays involving BChE digestion with subsequent assay of nonapeptide fragments facilitate identification of aged or nonaged adduct at the phosphylated serine-198 residue; therefore they are potentially useful in detecting agents such

as soman. Few methods have been published that use the assay of adducts to biomolecules to verify chemical warfare agent exposure in humans (Table 22-4).

Cholinesterase Analysis

OP chemical warfare agents are potent and irreversible inhibitors. Exposure results in excessive accumulation of acetylcholine that hyperstimulates cholinergic tissues and organs and ultimately leads to life-threatening cholinergic crises in humans.[3] The mechanism of OP toxicity is the inhibition of AChE and BChE involved in the termination of neurotransmission in cholinergic synapses and neuromuscular junctions of the central nervous system.[46] Synaptic AChE is not amenable to direct measurement, but because of functional similarities between synaptic and erythrocyte AChE, the activity of AChE in whole blood can be used as a reliable surrogate biomarker of central and peripheral nervous system activity.[47] Exposure to OP nerve agents, carbamates, pesticides, anesthetics, and drugs (such as cocaine) selectively reduces AChE and BChE activity.[3] Thus, it is crucial to diagnose OP exposure or intoxication early, and blood ChE activity (usually RBC-AChE) can be exploited as a tool for confirming exposure to these agents and commencing antidotal (oxime) therapy.[48,49] Because these ChE inhibitors comprise a group of structurally diverse compounds with a wide range of relative specificities for RBC-AChE and plasma BChE, a complete profile of inhibition is probably more accurately reflected if both ChEs are measured.

Exposure to OP nerve agents or pesticides that results in inhibition of less than about 20% AChE or BChE (especially if clinical symptoms are absent) may not easily be detected because of considerable inter- and intraindividual variations in AChE and (especially) BChE activities.[50] Moderate clinical symptoms of poisoning will be apparent at 50% to 70% AChE inhibition, with severe toxicity seen at greater than 90% inhibition.[51] While general measurement of ChE activity in blood is not specific for exposure to any OP nerve agent, carbamate, or pesticide, laboratory measurements by MS techniques can positively

TABLE 22-4

METHODS USED TO CONFIRM HUMAN EXPOSURES TO NERVE AGENT ADDUCTS TO BIOMOLECULES

Agent/Incident	Sample Matrix	Product Identified	Concentration Reported	Analytical Method
GB, Matsumoto and Tokyo, Japan	Serum	GB	Matsumoto (1.8–2.7 ng/mL) Tokyo (0.2–4.1 ng/mL)	GC-NPD[1,2]
GB, Tokyo, Japan	Red Blood Cell	IMPA, MPA	NR	GC-MS[3,4]
GB, Tokyo, Japan	Brain (cerebellum)	MPA	NR	GC-MS[5]
GB, Tokyo, Japan (selected samples)	Plasma/Serum	Phosphylated nonapeptides from BChE	10–20 pmol inhibited BChE/mL	LC-MS-MS[6]

BChE: butyrylcholinesterase
GB: sarin
GC: gas chromatography
IMPA: isopropyl methylphosphonic acid
LC: liquid chromatography
MPA: methylphosphonic acid
MS: mass spectrometric
NPD: nitrogen-phosphorus detector
NR: not reported

Data sources: (1) Polhuijs M, Langenberg JP, Benschop HP. New method for retrospective detection of exposure to organophosphorus anticholinesterases: application to alleged sarin victims of Japanese terrorists. *Toxicol Appl Pharmacol.* 1997;146:156–161. (2) Polhuijs M, Langenberg JP, Noort D, Hulst AG, Benschop HP. Retrospective detection of exposure to organophosphates: analyses in blood of human beings and rhesus monkeys. In: Sohns T, Voicu VA, eds. *NBC Risks: Current Capabilities and Future Perspectives for Protection*. Dordrecht, Holland, the Netherlands: Kluwer Academic Publishers; 1999:513–521. (3) Nagao M, Takatori T, Matsuda Y, et al. Detection of sarin hydrolysis products from sarin-like organophosphorus agent-exposed human erythrocytes. *J Chromatogr B Biomed Sci Appl.* 1997;701:9–17. (4) Nagao M, Takatori T, Matsuda Y, Nakajima M, Iwase H, Iwadate K. Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. *Toxicol Appl Pharmacol.* 1997;144:198–203. (5) Matsuda Y, Nagao M, Takatori T, et al. Detection of the sarin hydrolysis product in formalin-fixed brain tissues of victims of the Tokyo subway terrorist attack. *Toxicol Appl Pharmacol.* 1998;150:310–320. (6) Fidder A, Hulst AG, Noort D, et al. Retrospective detection of exposure to organophosphorus anti-cholinesterases: mass spectrometric analysis of phosphylated human butyrylcholinesterase. *Chem Res Toxicol.* 2002;15:582–590.

identify many OPs by evaluating their leaving group from fluoride-reactivated proteins.[28] Thus, the determination of an individual's ChE status can be important prior to decisions regarding oxime therapy and confirmation of OP poisoning, particularly in the case of long-lasting (eg, VX), rapidly aging (eg, soman) nerve agents and the surprising persistence of soman in blood and tissues.[52]

Although plasma BChE activity is measured in occupational and clinical toxicology laboratories, it should be noted that BChE exhibits different kinetic properties with nerve agents and pesticides than does RBC-AChE.[53] In humans, pesticide toxicity is well documented,[54] and completely different profiles of RBC-AChE and plasma BChE activities in pesticide-poisoned individuals have been reported.[55] Because decreased serum BChE often precedes a decline in RBC-AChE, an assay that measures both ChEs is more valuable for detecting initial (and smaller) changes in ChE levels that may signify exposure.

Several sensitive and specific assays for measuring AChE activity in blood have been developed for use in clinical and toxicology laboratories. For routine use, however, a number of drawbacks are apparent, including time-consuming sample preparation and long turn-around times. There is also a lack of standardization because of the difficulty comparing results between laboratories that use different ChE assays and report values in different or nonstandard units. None of the widely used methods has been approved by the US Food and Drug Administration (FDA). Clinical determination of AChE and BChE activities in blood commonly uses several techniques (colorimetric, electrometric, and radiometric) and normally measures either RBC-AChE or serum BChE concentrations, but usually not both.[56]

Colorimetric ChE Assays in the Clinical Laboratory

Several ChE assays are based on the enzyme-linked production of colored products. For these assays, monitoring color production as a function of time directly reflects enzyme activity or the ability of the enzyme to turn over substrate. Decreased enzyme turnover is reflected in less color production per unit time.

Ellman Assay. The Ellman method[57] is a popular colorimetric procedure for detecting and monitoring pesticide exposure. The breakdown of thiocholine substrates (acetylthiocholine and butyrylthiocholine) by AChE and BChE is detected kinetically using the Ellman reagent DTNB (5,5′-dithio-bis-2-nitrobenzoate). This assay is accurate, reliable, and inexpensive. However, the absorption maxima (412 nm) of the resulting yellow TNB^- (3-carboxy-4-nitrobenzenethiolate dianion) coincides with the Soret band of hemoglobin, resulting in interference and reduced assay sensitivity. By increasing the wavelength from 412 nm to 436 nm, hemoglobin interference can be reduced by 75% and assay sensitivity improved without significant sacrifice of the indicator (TNB^-) absorption.[58] Additionally, the molar extinction coefficient for TNB ($13.6 \times 10^3\ M^{-1}\ cm^{-1}$) used in the original Ellman assay[57] has been widely applied to calculate ChE activities, however, this value may vary depending on temperature, wavelength, and buffer conditions.[59] Changes in these experimental parameters can alter the extinction coefficient, resulting in different ChE activities from various laboratories. There are many published variations of the original cuvette-based Ellman assay, including the 96-well microtiter plate format.[60]

Walter Reed Army Institute of Research Whole Blood Assay. An important variation of the Ellman ChE assay is the Walter Reed Army Institute of Research Whole Blood Assay,[61] which uses 4,4′-dithiopyridine instead of DTNB as a chromogenic indicator and three thiocholine substrates (acetyl-, butyryl-, and propionyl-thiocholine). The absorption maxima in the ultraviolet range (324 nm) of the 4-thiopyridone formed yields a high signal-to-noise ratio because hemoglobin interference is minimal.[62] The use of three substrates in this assay rapidly and simultaneously provides redundancy and independent measurement of the RBC-AChE activities and plasma BChE in a small sample of unprocessed whole blood, using a 96-well microtiter plate spectrophotometer. The method is not labor intensive, and although it can be performed manually, it has been semi-automated using a Beckman-Coulter robotic platform for high sample throughput. A unique feature of the Walter Reed Army Institute of Research method is that the blood is not treated prior to assay (Figure 22-3). Thus, both AChE and BChE activities can be obtained without centrifugation or the use of inhibitors for whole, frozen, or lysed blood specimens.[62]

Test-Mate Assay. In addition to the laboratory-based methods, a field-deployable test unit is commercially available, the Test-Mate ChE system (EQM Research Inc, Cincinnati, OH). Further information (instructions, description, clinical trial date, etc) on the kit is available through the manufacturer.[63] The method is also based on the Ellman procedure and is supplied as a kit containing reagents to measure erythrocyte AChE and plasma BChE separately using a battery-operated photometric analyzer.[64] A specific serum BChE inhibitor (As1397, or 10-[α-diethylaminopropionyl]-phenothiazone) is included in the kit and is required to measure AChE (after a period of incubation). Two capillary tubes containing whole blood samples are

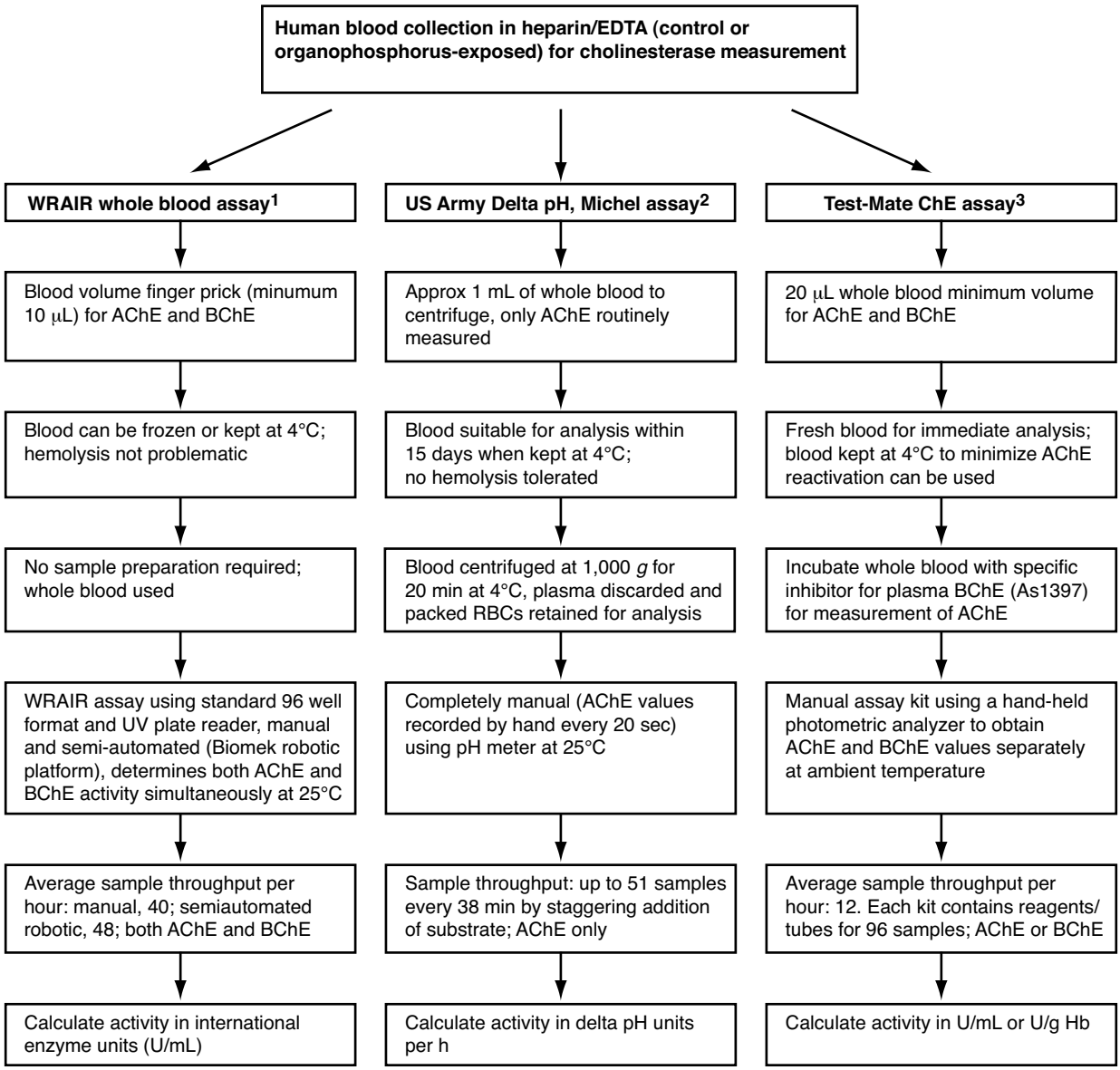


Fig. 22-3: Laboratory and field cholinesterase assays routinely used by the US Army and their required blood processing.
AChE: acetylcholinesterase
As1397: 10-(α-diethylaminopropionyl)-phenothiazone
BChE: butyrylcholinesterase
ChE: cholinesterase
EDTA: ethylenediaminetetraacetic acid
g: gravity
Hb: hemoglobin
RBCs: red blood cells
UV: ultraviolet
Data sources: (1) Gordon RK, Haigh JR, Garcia GE, et al. Oral administration of pyridostigmine bromide and huperzine A protects human whole blood cholinesterases from ex vivo exposure to soman. *Chemico Biol Interact.* 2005;157–158:239–246. (2) Ellin RI, Burkhardt BH, Hart RD. A time-modified method for measuring red blood cell cholinesterase activity. *Arch Environ Health.* 1973;27:48–49. (3) Taylor PW, Lukey BJ, Clark CR, Lee RB, Roussel RR. Field verification of Test-mate ChE. *Mil Med.* 2003;168:314–319.

necessary for AChE and BChE determination (and correction for hemoglobin content, which is also measured in each blood sample; see Figure 22-3). The kit is easy to use by a relatively untrained operator and matches the sensitivity of the laboratory-based Ellman methods, but has relatively low throughput because it is performed manually. Although the Test-Mate ChE kit is designed primarily for field use, where it is widely used for monitoring pesticide exposure in agricultural workers, longer processing times are required for complete AChE and BChE screening and hemoglobin measurement.

Electrometric ChE Assay (delta pH)

A manual method still widely used by the US Department of Defense to measure RBC-AChE is a modification[65] of the end-point delta pH method originally described by Michel.[66] This assay monitors the decrease in pH (using a simple pH electrode and pH meter) that occurs when AChE catalyzes the hydrolysis of acetylcholine to choline and acetic acid. The assay is initiated by the addition of substrate (acetylcholine) and the change in pH is monitored over 17 minutes. This method is slow and laborious, although throughput can be increased by staggering addition of substrate to each sample (see Figure 22-3). Up to 51 blood samples can be analyzed in 38 minutes, and the RBC-AChE activity is reported as a change (delta) in pH units per hour.[67] However, the delta pH method requires centrifugation of blood to pellet RBCs, followed by removal of the plasma (containing BChE) prior to analyzing AChE activity. Lysis of the blood sample is precluded, so blood must be iced (not frozen) before centrifugation and AChE analysis. Furthermore, because plasma BChE is not determined, the complete spectrum of blood inhibition is unknown. Although this technique was developed nearly 60 years ago, it is reliable, and the US Army (through the Department of Defense Cholinesterase Reference Laboratory) has a quality assurance testing program for primary RBC-AChE monitoring of more than 25,000 military personnel per year.[68]

Pretreatment Therapy for Nerve Agent Poisoning: Protection and Sequestering of Cholinesterase

Pyridostigmine Bromide. The US Army's current pretreatment against potential nerve agent poisoning is the reversible and fairly short-acting AChE inhibitor pyridostigmine bromide (PB), which was reviewed earlier.[69] PB is a quaternary ammonium compound that provides temporary protection (by carbamylation) of peripheral tissue and RBC-AChE. However, PB does not penetrate the blood-brain barrier, and thus does not afford protection against seizures and subsequent neuropathological states induced by a nerve agent such as soman.[70] During the Gulf War (1990–1991), more than 100,000 US and allied troops received PB (a single oral dose of 30 mg given every 8 h) as a pretreatment against exposure to soman. This method of pretreatment was demonstrated[62] in an FDA-supported clinical trial of human volunteers given PB as a single 30-mg dose (Table 22-5). Maximal RBC-AChE inhibition of about 27% was seen after 2.5 hours, with recovery of activity to almost 100% after 24 hours. To demonstrate protection and sequestering of AChE, the volunteers' PB-pretreated blood was exposed ex vivo to soman, followed by PB and soman removal from the blood using a small spin column, and monitoring the recovery of RBC-AChE (decarbamylation) (Figure 22-4). All of the AChE activity protected by PB pretreatment was restored to control levels within 3 hours. Plasma BChE was also inhibited in the same volunteers, albeit to a lesser extent (approximately 11%, about one third of the inhibition observed for AChE).[62] Although it was formally approved by the FDA as a specific pretreatment for soman poisoning in February 2003,[71] PB was not ordered to be taken by troops during the 2003 Iraq war.

Huperzine A: Potential Next Generation Peripherally and Centrally Acting Protection and Sequestering of Cholinesterase. Another pretreatment drug, physostigmine, can cross the blood-brain barrier and protect brain ChE in addition to RBC-AChE. However, psychological and behavioral side effects (although partially offset by a low dose of scopolamine to block muscarinic cholinergic receptors) preclude its use as an effective pretreatment against OP exposure. Furthermore, its use as an Alzheimer's drug to improve short-term memory has been discontinued because of multiple unwanted side effects, including nausea, dizziness, headaches, and sweating.

In contrast to PB, huperzine A (Hup A), an alkaloid isolated from the moss *Huperzia serrata*, is a reversible AChE inhibitor in both the peripheral and central nervous systems.[72,73] Hup A has been shown to be superior to physostigmine in its anti-ChE activity and has a longer biological half-life in humans and animals. Hup A is currently undergoing extensive clinical trials in the United States as a drug for Alzheimer's disease (it is already used for this purpose in China), though it is already sold as an over-the-counter nutraceutical supplement for memory enhancement (FDA approval is not required for this type of sale). Pretreatment with Hup A (in addition to postexposure treatment

TABLE 22-5

RED BLOOD CELL AND ACETYLCHOLINESTERASE PROTECTION STUDIES USING PYRIDOSTIGMINE BROMIDE AND HUPERZINE A AFTER EX-VIVO EXPOSURE TO SOMAN

Procedure*†	Techniques and Rationale
1) Obtain blood samples from human volunteers	Aliquot blood from subjects administered oral dose of 30 mg pyridostigmine bromide, 200 μg huperzine A, or no drug
2) Ex-vivo exposure to soman	Incubated blood with 1 μM soman for 10 min at room temperature; incubate control samples (no soman) with saline
3) Remove free drug and soman	Centrifuge samples through a C_{18} chromatography spin column to bind drug and remove from the blood
4) Allow time for AChE decarbamylation (PB) or dissociation (Hup A)	Maintain postcolumn samples at room temperature for up to 24 hours
5) Monitor time for regeneration of AChE after decarbamylation of PB or reversible inhibition by Hup A	Aliquot samples collected for AChE activity assay at indicated times postcolumn
6) Measure AChE activity	Use WRAIR whole blood ChE assay to determine recovered enzyme activity

*Acetylcholinesterase and pyridostigmine bromide or huperzine A yield an acetylcholinesterase-drug complex in procedures 1 and 2. This reaction demonstrates sequestered enzyme, which temporarily inhibits the active site.
†Over time, the acetylcholinesterase-drug complex becomes acetylcholinesterase plus pyridostigmine bromide (the decarbamylated form of acetylcholinesterase) or dissociated huperzine A in procedures 3 and 4. These reactions demonstrate the sequestered and then restored enzyme activity.
AChE: acetylcholinesterase
ChE: cholinesterase
PB: pyridostigmine bromide
Hup A: huperzine A

with atropine and an oxime) may represent a superior treatment strategy for protection against chemical warfare agent exposure and should be investigated further.

Animal studies have been used to examine the efficacy of high doses of Hup A against OP nerve agent toxicity. A dose of Hup A (500 μg/kg) significantly reduces soman lethality (protective ratios of 2–3 when used alone) and inhibits blood and brain AChE by 60% to 70%.[74] Unlike PB, Hup A can cross the blood-brain barrier and in rats protects brain AChE from inhibition by soman, thus preventing build-up of excessive acetylcholine leading to seizures and associated neuropathological damage. In guinea pig hippocampus, natural Hup A in subchronic doses (yielding 20%–30% inhibition of RBC-AChE) has little or no affinity for muscarinic, nicotinic, or *N*-methyl-D-aspartate excitatory amino acid receptors, and does not induce neuropathological damage.

Because Hup A reversibly binds to AChE, thus protecting the enzyme from reaction with OPs, the activity of the Hup-A–protected but inhibited AChE is restored once the drug-AChE complex spontaneously dissociates, which occurs after soman is cleared from the blood. To illustrate this, Hup-A– inhibited blood (drawn 1.5 h after human volunteers were given a dose of 200 μg Hup A; Figure 22-5) was exposed ex vivo to soman.[62] Blood samples were then rapidly centrifuged through a small column to remove any free soman and Hup A, the latter binding to the column matrix while allowing AChE and BChE to pass through the column. Under these circumstances, any RBC-AChE not protected by Hup A would be irreversibly inhibited by soman. In contrast, the RBC-AChE protected by Hup A would dissociate over time, and the AChE activity would eventually be restored. In one study, after soman treatment, no AChE activity was observed by the Walter Reed Army Institute of Research Whole Blood Assay in either the placebo- or drug-treated volunteers (see Figure 22-5). After the spin column removal of free Hup A and soman, and a 4-hour period to allow for complete dissociation, the Hup-A–inhibited AChE was restored to the level that was initially inhibited by the drug (about 60% inhibition by the drug before the column compared to 54% returned AChE activity after the column). AChE inhibition and sequestering

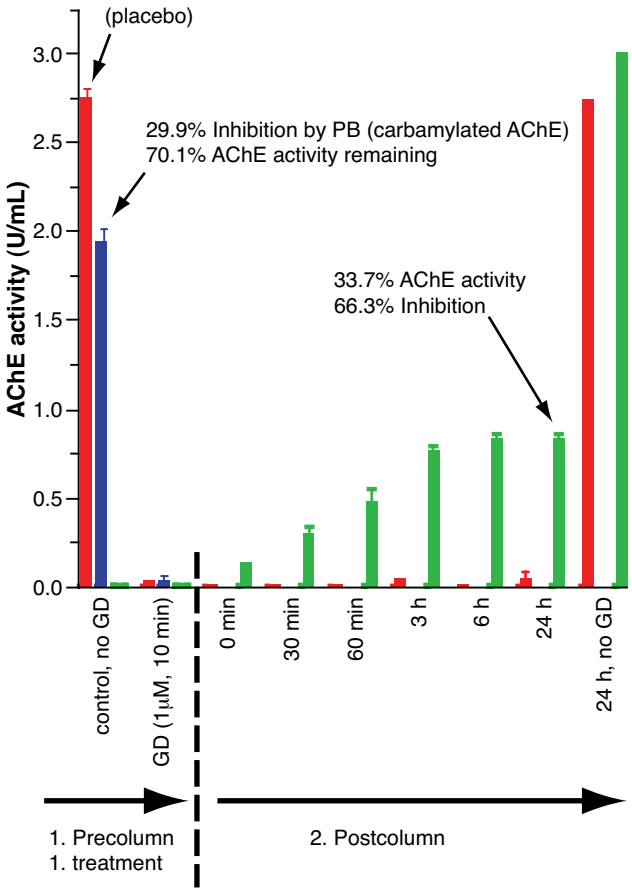


Fig. 22-4. Effects of soman (GD) on acetylcholinesterase (AChE) activity in whole blood from human volunteers who had taken pyridostigmine bromide (PB; 30 mg tablet). Blood was drawn 2.5 hours after dosing, when red blood cell (RBC) AChE is maximally inhibited by PB (blue bar, left of dashed line). After GD exposure, PB and GD were removed using a C_{18} chromatography spin column (postcolumn treatment). The PB-protected (carbamylated and sequestered) AChE activity returned by 6 hours postcolumn. Green bars show the correlation between the initial percent inhibition of RBC-AChE by PB (solid red line) and the subsequent return of AChE activity due to decarbamylation of the protected enzyme after removing PB and GD (green bars). While there was about 29.9% inhibition of RBC-AChE by PB before GD exposure (blue bar with arrow), at 24 hours (the green bar with arrow) there was about 33.7% return in activity after the column, demonstrating protection of RBC-AChE by PB pretreatment. Error bars represent the mean plus or minus the standard error of the mean.
AChE: acetylcholinesterase
GD: soman
PB: pyridostigmine bromide
RBC: red blood cell

by Hup A increased, compared to PB. Thus, Hup A is highly effective in protecting RBC-AChE from ex-vivo soman exposure.

As a pretreatment, Hup A is a specific and highly selective RBC-AChE inhibitor, with serum BChE remaining unaffected at physiological concentrations.[75] Thus, after exposure to an OP nerve agent, the effective bioscavenging capacity for serum BChE is preserved.[76] This lack of BChE inhibition represents an additional advantage of Hup A in preventing OP toxicity, and helps explain increased tolerance of Hup-A–pretreated animals to soman in comparison to animals pretreated with PB.

Studies of toxicology and treatment for nerve agent exposure have predominantly focused on lethal and supralethal doses of the agent. The acute and long-term effects of sarin in humans were well documented following the terrorist attacks in Japan in 1994 and 1995. Several severely poisoned victims (in cardiopulmonary arrest or in comas with generalized convulsions) had plasma BChE activities of 20% of normal (80% inhibited).[77] However, information regarding the delayed or long-term subclinical effects of low-level or trace amounts of sarin and other nerve agents, insecticides, and a variety of environmental chemicals (to which individuals may be asymptomatic) is relatively scarce, both in military and civilian environments. Potential scenarios can be envisioned in which low or trace exposures become significant. Given the possibility of urban terrorism involving chemical warfare OP agents, federal, state, and local authorities now have a variety of sensitive and accurate ChE and OP detection assays to initiate appropriate containment, decontamination, and treatment measures.

SULFUR MUSTARD

Background

Analysis of specimens, such as blood or urine, following a suspected chemical warfare agent exposure has been rare. Historically, biomedical samples collected after a suspected exposure were used for standard clinical assays and not preserved for later analysis. Highly sophisticated analytical methods required to verify chemical warfare agent exposure were not a part of the clinical laboratory methods inventory. More

recently, a large portion of the biomedical samples obtained from casualties of suspected exposure to sulfur mustard have come from the Iran-Iraq war in the 1980s. Many victims of that conflict who exhibited clinical signs consistent with exposure to sulfur mustard were transported to hospitals in Europe for medical treatment. Prior to 1995 laboratory analysis of these specimens used methods to measure unmetabolized sulfur mustard or thiodiglycol (TDG), a hydrolysis product of sulfur mustard. Fortunately, some of the blood components and urine specimens were frozen and reanalyzed years later, after newer analytical methods were developed. Recently reported methods of analysis generally indicate greater levels of sensitivity than previous methods or target other biomarkers of sulfur mustard exposure. In addition to samples from

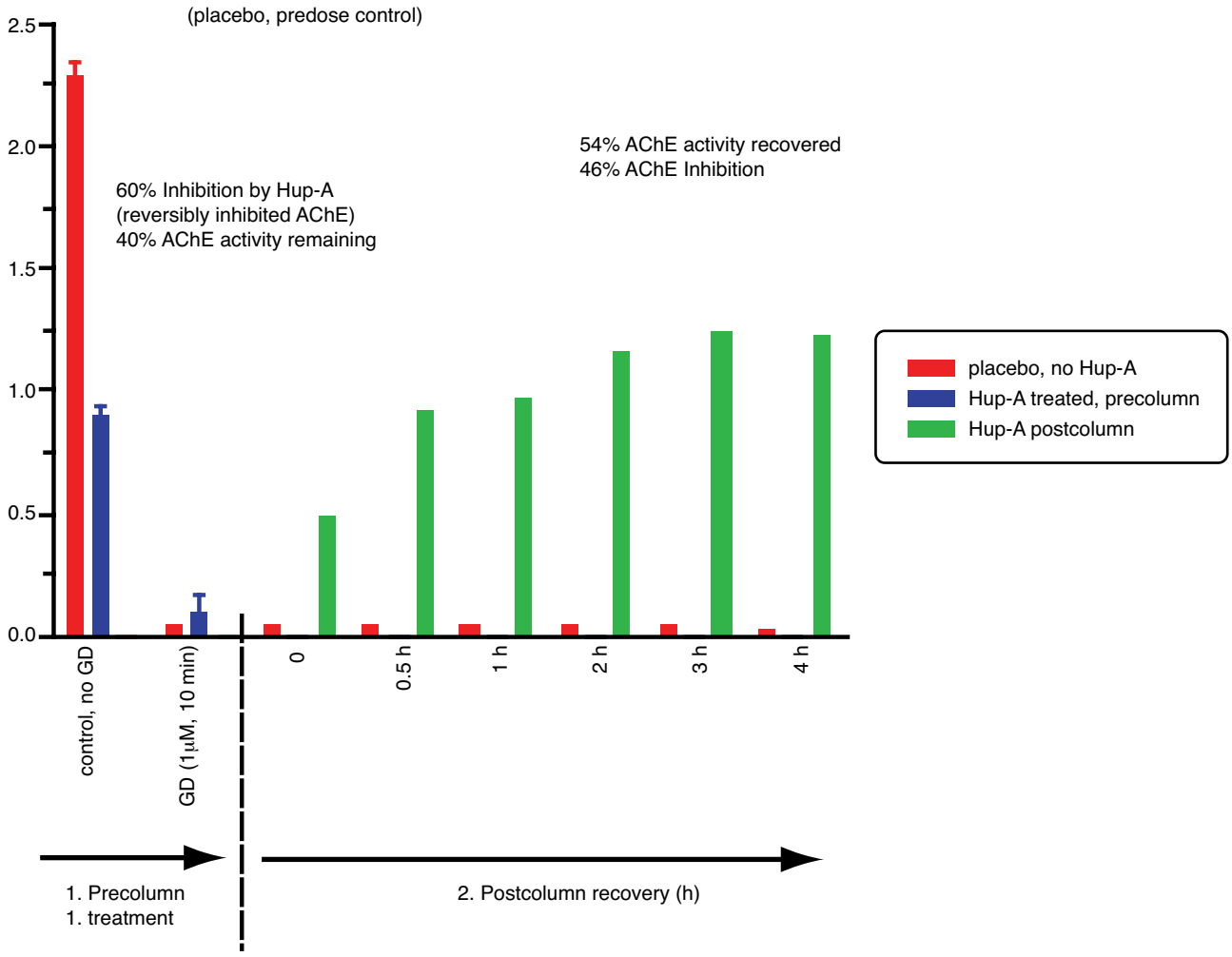


Fig. 22-5. Effects of soman (GD) on acetylcholinesterase (AChE) activity in whole blood from human volunteers who were given an increasing dose of huperzine A (Hup A). Blood was drawn 1.5 hours after the final 200-μg dose (blue bar, left of dashed line). The red bars represent red blood cell AChE from volunteers prior to receiving a placebo, while the blue bars represent AChE from an individual receiving the 200-μg Hup A dose. In the first part, after soman treatment, no AChE activity is observed by the Walter Reed Army Institute of Research assay in either the placebo- or drug-treated volunteers (red bars, close to 0 U/mL). However, after the spin column removal of free Hup A and soman, and a 4-hour period to allow for complete dissociation, the Hup-A–inhibited AChE is restored to the level that was initially inhibited by the drug (green bar with arrow, about 60% inhibition by the drug before the column versus 54% returned AChE activity after the column). Error bars represent the mean plus or minus the standard error of the mean. Note the increased inhibition and sequestering of AChE by Hup A compared to pyridostigmine bromide (see Fig. 22-4).
AChE: acetylcholinesterase
Hup A: huperzine A

the Iran-Iraq war, a small number of samples from individuals exposed to sulfur mustard in laboratory and field situations were collected, stored, and analyzed using more recently developed methods.

Some of sulfur mustard's physical properties and biochemical reactions are addressed in this volume (see Chapter 8, Vesicants) and have been reviewed extensively elsewhere.[78] Of primary importance in the development of assays for sulfur mustard is the formation of a highly reactive sulfonium ion that is produced following cyclization of an ethylene group of sulfur mustard. The sulfonium ion readily reacts with nucleophiles, such as water, or combines with a variety of nucleophilic sites in macromolecules. The resulting chemical reactions are able to produce a number of free metabolites and stable adducts that can be exploited for analysis in blood, urine, and tissue samples.[7,79,80] This section focuses primarily on metabolites that have been identified in biomedical samples from sulfur mustard casualties.

Before the early 1990s analytical methods for verifying exposure to sulfur mustard consisted of assays for the unmetabolized compound or the hydrolysis product TDG. Since 1995 a number of significant advances have occurred. Many new metabolites have been identified from specimens of sulfur-mustard–exposed individuals, and instrument advances, such as the ability to interface LC with MS and the use of tandem MS, have resulted in significant increases in test sensitivity and selectivity. Most newer methods of verifying exposure to sulfur mustard require extensive sample processing prior to introduction into the analytical system. The use of MS has enabled the incorporation of isotopically labeled forms of analytes for use as internal standards during the earliest stages of sample preparation. This has resulted in greater reproducibility of assays and made them more amenable to quantitative analysis. Although the laboratory methods presented in this section are not considered routine or standard, the efforts by a small number of laboratories worldwide that are active in this area of research have made the methods more attainable to a wider range of laboratories.

Analysis of Urine Samples

There are currently five urinary metabolites of primary interest in verifying exposure to sulfur mustard. Two of the metabolites, TDG and thiodiglycol sulfoxide (TDG-sulfoxide), are derived from chemical hydrolysis reactions (Figure 22-6). The other three products are formed following sulfur mustard's reaction with glutathione (GSH). Each of the five analytes has been identified in the urine of sulfur-mustard–exposed individuals.

Analytical Methods

Efforts to analyze specific biomarkers of sulfur mustard exposure in urine samples prior to 1995 targeted either unmetabolized sulfur mustard or TDG (Table 22-6). Vycudilik prepared urine samples by initially saturating them with sodium chloride followed by organic extraction using diethylether. The organic portion was evaporated under nitrogen and reconstituted with methylene chloride. After the addition of silica gel, the methylene chloride was evaporated under nitrogen, reconstituted with solvent, and analyzed using GC-MS.[81] Vycudilik later modified the method to isolate possible conjugates of sulfur mustard.[82] The primary difference in the latter study was the addition of strong acid to the urine samples. Urine samples were mixed with an equal amount of concentrated hydrochloric acid and saturated with sodium chloride. Using steam distillation, the distillate was collected in ether. Following sodium chloride saturation of the aqueous layer, the ether layer was dried and the residue dissolved in methylene chloride and silica gel. Samples were analyzed using GC and high-resolution MS. Vycudilik reported that the methods could not distinguish between sulfur mustard and its hydroxyethyl metabolites present in the urine samples.[82]

Wils et al treated urine with concentrated hydrochloric acid to convert TDG back to sulfur mustard.[83,84] Two methods were reported, only the later method is described here. The urine was passed through two C18 solid phase extraction cartridges. Next, a solution

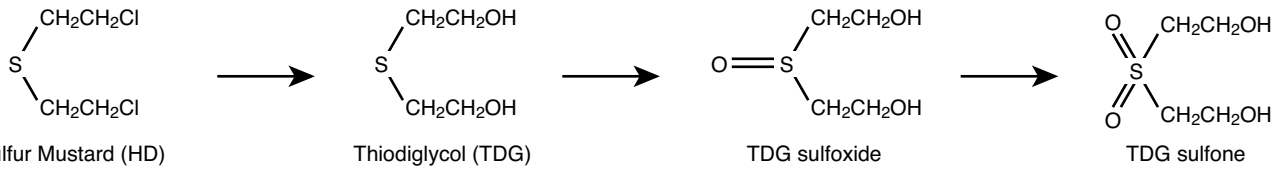


Fig. 22-6. Hydrolysis of sulfur mustard to produce thiodiglycol, followed by oxidation reactions.
HD: sulfur mustard
TDG: thiodiglycol

of deuterated TDG was passed through the same cartridges. The purified urine was mixed with concentrated hydrogen chloride (HCl) and heated. Sulfur mustard was purged from the solution and trapped onto a Tenax-TA adsorption tube. Analysis was performed using a thermodesorption cold trap injector interfaced with GC-MS. Analysis of urine samples obtained from a control group of patients found levels of TDG at low nanogram-to-milliliter concentrations. While most of the control levels were approximately 5 ng/mL, two individuals had levels that exceeded 20 ng/mL. These high background levels probably indicate that the method was also converting another analyte, in addition to TDG, into sulfur mustard (see below).

TABLE 22-6

REPORTS PUBLISHED PRIOR TO 1995 SHOWING LABORATORY ANALYSIS OF HUMAN BIOMEDICAL SAMPLES FOLLOWING SUSPECTED EXPOSURE TO SULFUR MUSTARD

Patient Sample Information*	Unmetabolized Sulfur Mustard	Hydrolysis Product†
Urine samples from 2 Iran-Iraq War casualties treated at Vienna hospital; collected 7 days after incident (no date given)[1]	Patient 1: 1.0 ng/mL Patient 2: 1.5 ng/mL	NM
Urine samples from 5 Iranian casualties treated at Ghent hospital; collected 10 days after incident (March 9, 1984)[2]	NM	Patient C1: 90 ng/mL Patient C2: 45 ng/mL Patient C3: 40 ng/mL Patient C4: 40 ng/mL Patient C5: 15 ng/mL Control samples: 3–55 ng/mL
Urine samples from 5 Iranian casualties treated at Utrecht hospital; collected 10 days after incident (March 9, 1984)[2]	NM	Patient range: 3–140 ng/mL Control samples: 3–55 ng/mL
Hair samples from 2 Iranian casualties; collected 1 day after incident (Feb 27, 1986)[3]	Patient 1: 0.5–1.0 μg/gram Patient 2: not detected	NM
Autopsy specimens (tissues and body fluids) from Iranian casualty treated at Munich hospital; collected 7 days after incident (1985)[4]	Urine: not detected; Fat, Skin, Brain, Kidney: 5–15 mg/kg; Muscle, Liver, Spleen, Lung: 1–2 mg/kg	NM
Urine samples from 12 Iran-Iraq War casualties (1986); no other details provided[5]	1–30 ng/mL for 6 individuals; not detected in 6 individuals	NM
Urine samples from 7 Iranian casualties treated at Ghent hospital; collected 5–6 and 18–19 days after incident (Feb 12–13, 1986)[6]	NM	5–6 days postexposure: range 7–336 ng/mL; 18–19 days postexposure: range 3–7 ng/mL
Urine samples from 3 Iranian casualties treated at Ghent hospital; collected 18–19 days after incident (Feb 12–13, 1986)[6]	NM	Patient range: 4–8 ng/mL Control samples: 1–21 ng/mL
Urine samples from 8 Iranian casualties treated at Utrecht hospital; collected 8–9 days after incident (Feb 12–13, 1986)[6]	NM	Patient range: 5–76 ng/mL Control samples: 1–21 ng/mL

*These are the known details of the incident and sample collection time after suspected exposure.
†The hydrolysis product was thiodiglycol.
NM: not measured

Data sources: (1) Vycudilik W. Detection of mustard gas bis(2-chloroethyl)-sulfide in urine. *Forensic Sci Int.* 1985;28:131–136. (2) Wils ERJ, Hulst AG, de Jong AL, Verweij A, Boter HL. Analysis of thiodiglycol in urine of victims of an alleged attack with mustard gas. *J Anal Toxicol.* 1985;9:254–257. (3) United Nations Security Council. *Report of the mission dispatched by the Secretary-General to investigate allegations of the use of chemical weapons in the conflict between the Islamic Republic of Iran and Iraq.* New York, NY: UN; 1986. Report S/17911. (4) Drasch G, Kretschmer E, Kauert G, von Meyer L. Concentrations of mustard gas [bis(2-chloroethyl)sulfide] in the tissues of a victim of a vesicant exposure. *J Forensic Sci.* 1987;32:1788–1793. (5) Vycudilik W. Detection of bis(2-chloroethyl)-sulfide (Yperite) in urine by high resolution gas chromatography-mass spectrometry. *Forensic Sci Int.* 1987;35:67–71. (6) Wils ERJ, Hulst AG, van Laar J. Analysis of thiodiglycol in urine of victims of an alleged attack with mustard gas, part II. *J Anal Toxicol.* 1988;12:15–19.

Drasch et al examined urine samples for unmetabolized sulfur mustard. Following organic extraction, thin-layer chromatography, and derivatization with gold, the extracts were analyzed using electrothermal atomic absorption spectroscopy.[85]

More recently, additional methods have been developed for trace level analysis of TDG and TDG-sulfoxide in urine.[86–89] There are a number of characteristics common to the methods (Table 22-7). They all use GC in association with some form of MS analysis, use a derivatizing agent to make the analyte more amenable to GC analysis and to increase sensitivity, and incorporate an isotopically labeled form of TDG as an internal standard. Most of the methods use a solid phase extraction cartridge for sample preparation. Some of the methods incubate the urine samples with glucuronidase with sulfatase activity to release any glucuronide-bound conjugates. Some of the methods use titanium trichloride in hydrochloric acid to reduce TDG-sulfoxide to TDG. The strong acid also hydrolyzes acid-labile esters of TDG and TDG-sulfoxide (Table 22-8). Ultimately, each of the methods converts all target analytes into the single analyte TDG for analysis. All of the methods have similar limits of detection, approximately 0.5 to 1 ng/mL. Although an assay has been developed to analyze TDG-sulfoxide separately without a conversion to TDG, the method is complicated by the high polarity of the analyte.[90] Consequently, the more common approach is to use the reducing agent titanium trichloride.

Unfortunately, regardless of the analytical method used, background levels have consistently been found in urine samples obtained from nonexposed individuals. In the most extensive study of background levels, urine samples from 105 individuals were examined for sulfur mustard metabolites using an assay that incorporates both a deconjugation process and a reduction step (ie, the assay measures free and bound forms of both TDG and of TDG-sulfoxide).[89] Quantifiable background levels were observed in 82% of the samples. Nearly 60% of the samples had observed levels of TDG in the 0.5- to 2.0-ng/mL range, while approximately 9% had levels in the 10- to 20-ng/mL range. When urine samples with higher background

TABLE 22-7

ANALYSIS METHODS FOR URINE SAMPLES TO MEASURE THIODIGLYCOL OR THIODIGLYCOL AND THIODIGLYCOL-SULFOXIDE

Instrumentation	Derivatizing Agent	SPE Cartridge	Glucuronidase Incubation	$TiCl_3$ Reduction	Internal Standard	Detection Limit
Negative ion chemical ionization GC-MS	Pentafluorobenzoyl chloride	Florisil	Yes	No	$^2H_4$-TDG	~ 1 ng/mL[1]
Electron impact GC-MS	Heptafluorobutyric anhydride	None	Yes	No	$^2H_8$-TDG	~ 1 ng/mL[2]
Negative ion chemical ionization GC-MS-MS	Pentafluorobenzoyl chloride	Florisil	No	Yes	$^2H_4$-TDG	< 1 ng/mL[3]
Positive chemical ionization GC-MS-MS	Heptafluorobutyric anhydride	Oasis HLB	Yes	Yes	$^{13}C_4$-TDG	0.5 ng/mL[4]

$^{13}C_4$: isotopically labeled carbon
$^2H_4$: isotopically labeled hydrogen or deuterium
$^2H_8$: isotopically labeled hydrogen or deuterium
GC: gas chromatography
HLB: hydrophilic-lipophilic-balanced
MS: mass spectrometry
SPE: solid phase extraction
TDG: thiodiglycol
$TiCl_3$: titanium trichloride
Data sources: (1) Black RM, Read RW. Detection of trace levels of thiodiglycol in blood, plasma and urine using gas chromatography-electron-capture negative-ion chemical ionisation mass spectrometry. *J Chromatogr.* 1988;449:261–270. (2) Jakubowski EM, Woodard CL, Mershon MM, Dolzine TW. Quantification of thiodiglycol in urine by electron ionization gas chromatography-mass spectrometry. *J Chromatogr.* 1990;528:184–190. (3) Black RM, Read RW. Improved methodology for the detection and quantitation of urinary metabolites of sulphur mustard using gas chromatography-tandem mass spectrometry. *J Chromatogr B Biomed Appl*. 1995;665:97–105. (4) Boyer AE, Ash D, Barr DB, et al. Quantitation of the sulfur mustard metabolites 1,1′-sulfonylbis[2-(methylthio)ethane] and thiodiglycol in urine using isotope-dilution gas chromatography-tandem mass spectrometry. *J Anal Toxicol*. 2004;28:327–332.

TABLE 22-8

TARGET ANALYTES FOR ANALYSIS METHODS OUTLINED IN TABLE 22-7

TDG			TDG-Sulfoxide		
Free	Glucuronide-bound conjugates	Acid-labile esters	Free	Glucuronide-bound conjugates	Acid-labile esters
Yes	Yes	No	No	No	No[1]
Yes	Yes	No	No	No	No[2]
Yes	No	Yes	Yes	No	Yes[3]
Yes	Yes	Yes	Yes	Yes	Yes[4]

TDG: thiodiglycol

Data sources: (1) Black RM, Read RW. Detection of trace levels of thiodiglycol in blood, plasma and urine using gas chromatography-electron-capture negative-ion chemical ionisation mass spectrometry. *J Chromatogr.* 1988;449:261–270. (2) Jakubowski EM, Woodard CL, Mershon MM, Dolzine TW. Quantification of thiodiglycol in urine by electron ionization gas chromatography-mass spectrometry. *J Chromatogr.* 1990;528:184–190. (3) Black RM, Read RW. Improved methodology for the detection and quantitation of urinary metabolites of sulphur mustard using gas chromatography-tandem mass spectrometry. *J Chromatogr B Biomed Appl*. 1995;665:97–105. (4) Boyer AE, Ash D, Barr DB, et al. Quantitation of the sulfur mustard metabolites 1,1′-sulfonylbis[2-(methylthio)ethane] and thiodiglycol in urine using isotope-dilution gas chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2004;28:327–332.

levels were reanalyzed without the reduction step, the TDG level in all the samples was less than 2.5 ng/mL. This indicates that the free and bound forms of the TDG-sulfoxide, rather than the free and bound forms of TDG, are responsible for a larger portion of the observed background levels in the nonexposed human urine samples. These results are consistent with those found in other, smaller studies of background levels.[86,87,90,91] Boyer et al discovered that storage condition of urine samples must also be considered when analyzing samples for TDG and TDG-sulfoxide.[89] In a study of urine samples stored at – 20°C for an 8-month period, they found that all free and conjugated TDG in the samples had oxidized to free and conjugated TDG-sulfoxide. Consequently, the use of a reducing agent was shown to be critical for the analysis of samples that had been frozen for any length of time.

Black et al identified a series of metabolites formed from sulfur mustard's reaction with GSH, a small-molecular–weight tripeptide that acts as a free radical scavenger (Figure 22-7).[91–93] While a large number of metabolites were identified in animal experiments, there are three verified reaction products in urine samples obtained from individuals exposed to sulfur mustard. One set of reaction products is believed to result from metabolism of the sulfur-mustard–GSH conjugate by the β-lyase enzyme (see Figure 22-7d). Two β-lyase metabolites have been identified in the urine from exposed individuals:

- 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTESE) and
- 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE).

MSMTESE and SBMSE can be reduced using titanium chloride and analyzed by GC-MS-MS as a single analyte: 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE; Exhibit 22-1).[94,95] Black et al reported a limit of detection of 0.1 ng/mL,[94] while Young et al extended the lower limit of detection to 0.038 ng/mL.[95] To date, no background levels have been found in the urine of unexposed individuals, including studies where urine samples from over 100 individuals were analyzed using two different assay methods.[89,95] Alternatively, MSMTESE and SBMSE can be analyzed individually without reducing the two analytes to single analyte using electrospray LC-MS-MS.[96] Lower limits of detection were 0.1 to 0.5 ng/mL for each of the analytes.

The final urinary biomarker to be discussed is also a reaction product of sulfur mustard with GSH: 1,1′-sulfonylbis[2-S-(N-acetylcysteinyl)ethane] (see Figure 22-7e; Table 22-9). Using solid phase extraction for sample cleanup and analyte concentration, followed by analysis with negative ion electrospray LC-MS-MS, Read and Black were able to achieve detection limits of 0.5 to 1.0 ng/mL.[97]

Assays for several other potential urinary analytes have been developed, but these analytes have yet to be confirmed in human-exposed samples. N7-(2-hydroxyethylthioethyl) guanine is a breakdown product from alkylated DNA that has been observed in animal studies. Fidder et al developed both a GC-MS method that requires derivatization of the analyte and an

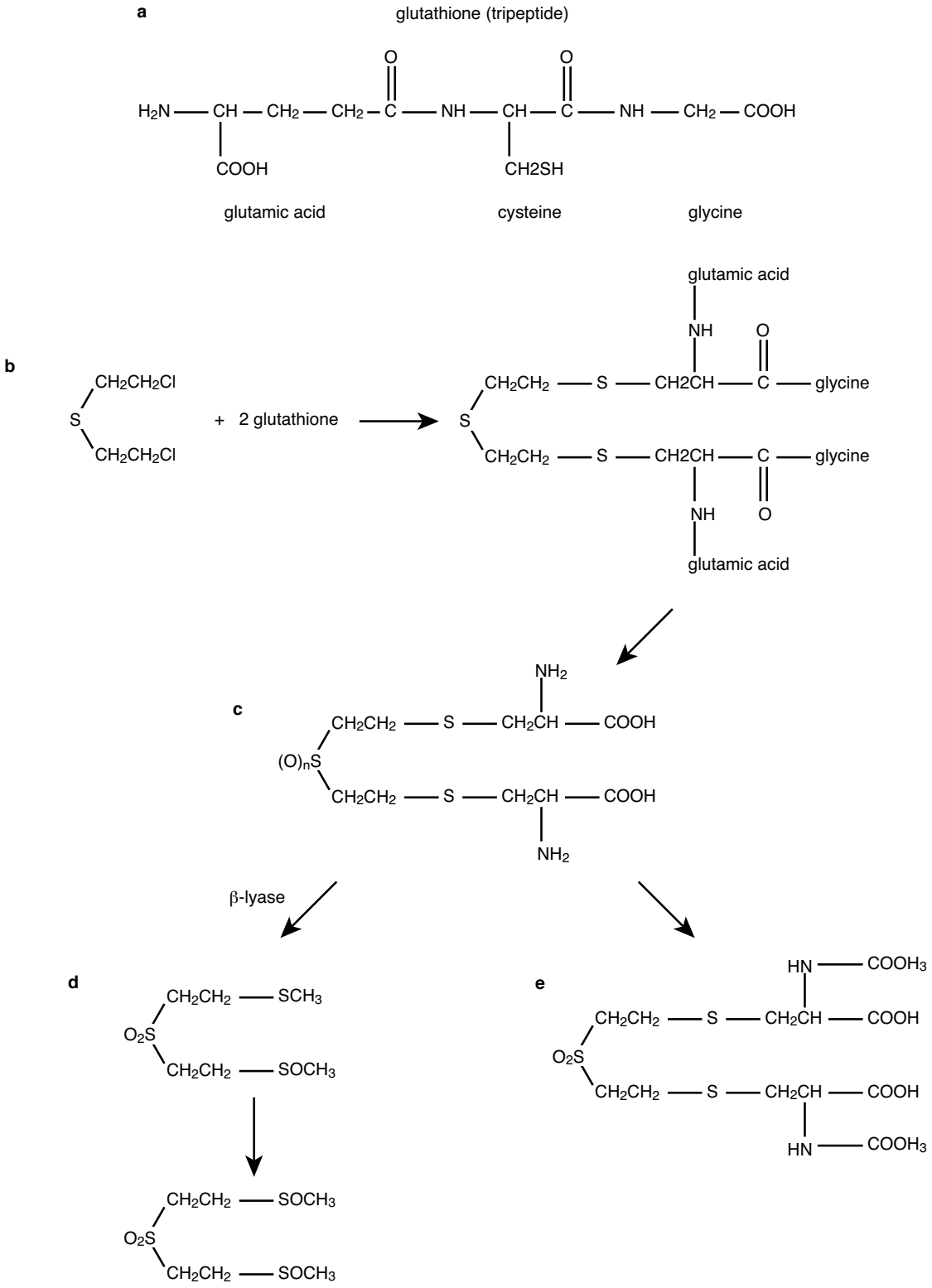


Fig. 22-7. Reaction pathway proposed by Black et al (1992). **(a)** Structure of glutathione. **(b)** Reaction of sulfur mustard and glutathione. **(c)** Intermediate product. **(d)** β-lyase metabolites 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSM-TESE) and 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE). **(e)** Bis-mercapturic acid conjugate of mustard sulfone.
Data source: Black RM, Brewster K, Clarke RJ, Hambrook JL, Harrison JM, Howells DJ. Biological fate of sulphur mustard, 1,1′-thiobis(2-chloroethane): isolation and identification of urinary metabolites following intraperitoneal administration to rat. *Xenobiotica*. 1992;22:405–418.

LC-MS-MS method that can analyze the compound directly.[98] Other possible urinary analytes are an imidazole derivative formed from sulfur mustard's reaction with protein histidine residues[99] and sulfur mustard adducts to metallothionien.[100]

Application to Human Exposure

Vycudilik analyzed urine samples from two casualties of the Iran-Iraq War who were brought to a hospital in Vienna, Austria, for treatment of suspected exposure to sulfur mustard.[81] The exposure was believed to have occurred 1 week prior to their arrival in Vienna. No clinical description of the patients' injuries was provided in the report. The concentration of sulfur mustard found in their urine samples using GC-MS was approximately 1.0 ng/mL and 1.5 ng/mL. Additional urine samples were obtained from the patients several days after admission to the hospital. Analysis using the same method produced negative results for all samples.

Vycudilik also analyzed urine samples obtained from 12 Iran-Iraq War casualties.[82] The only clinical description of the patients was the observation that they had severe skin lesions resulting from an alleged mustard gas attack. Urine samples from six of the patients produced positive results for sulfur mustard. Concentrations found ranged from 1 to 30 ng/mL. The method could not distinguish between sulfur mustard or its hydroxyethyl metabolites present in the urine samples.

Wils et al examined a large number of urine samples for TDG concentrations. The samples were obtained from Iranian casualties of the Iran-Iraq War transported to western European hospitals in Ghent and Utrecht for treatment.[83,84] The majority of the urine samples

EXHIBIT 22-1

SAMPLE PREPARATION METHODS FOR THE GAS CHROMATOGRAPHIC/MASS SPECTROMETRIC/MASS SPECTROMETRIC ANALYSIS OF THE SULFUR MUSTARD URINARY β-LYASE METABOLITES

Procedure of Black et al:

- Add the internal standard deuterated 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] to 1 mL of urine.
- Add 0.4 mL of titanium trichloride.
- Incubate sample at 40°C overnight (16 hours).
- Filter solution through a preconditioned C_8 Bond Elut solid-phase extraction cartridge.
- Wash cartridge with water followed by a methanol and water mixture.
- Allow cartridge to dry.
- Elute analytes with acetone.
- Evaporate to dryness under nitrogen and dissolve in toluene.
- Analyze using GC-MS-MS with ammonia chemical ionization.[1]

Procedure of Young et al:

- Place 0.5 mL of urine into a 15 mL tube.
- Add the internal standard ^{13}C-1,1′-sulfonylbis[2-(methythio)ethane] to the urine.
- Add 1 mL of titanium trichloride.
- Incubate sample at 75°C for 1 hour.
- Add 2 mL of 6N sodium hydroxide and mix.
- Centrifuge samples for 5 minutes.
- Pour supernatant into a Chem Elut column.
- Elute analytes with 16 mL of dichloromethane and acetonitrile mixture.
- Evaporate to dryness under nitrogen and dissolve in toluene.
- Analyze using GC-MS-MS with isobutane chemical ionization.[2]

GC: gas chromatography
MS: mass spectrometry
Data sources: (1) Black RM, Clarke RJ, Read RW. Analysis of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane, metabolites of sulphur mustard, in urine using gas chromatography-mass spectrometry. *J Chromatogr.* 1991;558:405–414. (2) Young CL, Ash D, Driskell WJ, et al. A rapid, sensitive method for the quantitation of specific metabolites of sulfur mustard in human urine using isotope-dilution gas chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2004;28:339–345.

TABLE 22-9

ANALYTICAL METHODS USED TO VERIFY EXPOSURE TO SULFUR MUSTARD IN BIOMEDICAL SAMPLES

Sample Matrix	Biomarker	Sample Preparation	Analytical Method	LOD
Urine, blood, plasma	TDG	Enzyme incubation, derivatization	Negative ion chemical ionization GC-MS	~ 1 ng/mL[1]
Urine	TDG	Enzyme incubation, derivatization	Electron impact GC-MS	~ 1 ng/mL[2]
Urine	TDG, TDG-sulfoxide	$TiCl_3$ reduction, derivatization	Negative ion chemical ionization GC-MS-MS	< 1 ng/mL[3]
Urine	TDG, TDG-sulfoxide	Enzyme incubation, $TiCl_3$ reduction, derivatization	Positive ion chemical ionization GC-MS	0.5 ng/mL[4]
Urine	TDG-sulfoxide	Derivatization	Negative ion chemical ionization GC-MS	2 ng/mL[5]
Urine	SBMTE	$TiCl_3$ reduction	Positive ion chemical ionization GC-MS-MS	0.1 ng/mL[6]
Urine	SBMTE	$TiCl_3$ reduction	Positive ion chemical ionization GC-MS-MS	0.04 ng/mL[7]
Urine	MSMTESE	SPE cartridge extraction	Positive ion electrospray LC-MS-MS	0.1–0.5 ng/mL[8]
Urine	SBMSE	SPE cartridge extraction	Positive ion electrospray LC-MS-MS	0.1–0.5 ng/mL[8]
Urine	Bis-(N-acetyl cysteine) conjugate	SPE cartridge extraction	Negative ion electrospray LC-MS-MS	0.5–1 ng/mL[9]
Blood	Hemoglobin valine adduct	Globin isolation, valine cleavage by Edman degradation, derivatization	Negative ion chemical ionization GC-MS	100 nM whole blood exposure[10,11]
Blood	Hemoglobin valine adduct	Globin isolation, valine cleavage by Edman degradation	High-resolution negative ion chemical ionization GC-MS	0.5 pmol adduct/mL[12]
Blood	Hemoglobin histidine adduct	Acid hydrolysis of globin, derivatization	Positive ion electrospray LC-MS-MS	Not reported[12]
Blood	Hemoglobin histidine adduct	Acid hydrolysis of globin, derivatization	Positive ion electrospray LC-MS-MS	10 μM whole blood exposure[13]
Plasma	Albumin cysteine adduct	Albumin isolation, pronase digestion	Positive ion electrospray LC-MS-MS	10 nM whole blood exposure[14,15]
Blood, plasma	Protein adducts	Protein precipitation, alkaline hydrolysis, derivatization, SPE extraction	Negative ion chemical ionization GC-MS	25 nM plasma exposure[16]
Blood	DNA adducts	WBC isolation, lysis, extraction, treatment with RNase and proteinase K	Immunuslotblot assay	50 nM whole blood exposure[17]
Skin	DNA adducts	Epidermal layer isolation, lysis, extraction, treatment with RNase and proteinase K	Immunuslotblot assay	1 sec skin exposure to saturated vapor[17]
Skin	Keratin adducts	Alkaline hydrolysis, derivatization	LC-radiometric detector	Not reported[18]

(**Table 22-9** *continues*)

Table 22-9 *continued*

Cl: chemical ionization
DNA: deoxyribonucleic acid
GC: gas chromatography
LC: liquid chromatography
LOD: limit of detection
MS: mass spectrometry
MSMTESE: 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane
RNase: ribonuclease
SBMSE: 1,1′-sulfonylbis[2-(methylsulfinyl)ethane]
SBMTE: 1,1′-sulfonylbis[2-(methylthio)ethane]
SPE: solid phase extraction
TDG: thiodiglycol
$TiCl_3$: titanium trichloride
WBC: white blood cell
Data sources: (1) Black RM, Read RW. Detection of trace levels of thiodiglycol in blood, plasma and urine using gas chromatography-electron-capture negative-ion chemical ionisation mass spectrometry. *J Chromatogr.* 1988;449:261–270. (2) Jakubowski EM, Woodard CL, Mershon MM, Dolzine TW. Quantification of thiodiglycol in urine by electron ionization gas chromatography-mass spectrometry. *J Chromatogr.* 1990;528:184–190. (3) Black RM, Read RW. Improved methodology for the detection and quantitation of urinary metabolites of sulphur mustard using gas chromatography-tandem mass spectrometry. *J Chromatogr B Biomed Appl*. 1995;665:97–105. (4) Boyer AE, Ash D, Barr DB, et al. Quantitation of the sulfur mustard metabolites 1,1′-sulfonylbis[2-(methylthio)ethane] and thiodiglycol in urine using isotope-dilution gas chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2004;28:327–332. (5) Black RM, Read RW. Methods for the analysis of thiodiglycol sulphoxide, a metabolite of sulphur mustard, in urine using gas chromatography-mass spectrometry. *J Chromatogr.* 1991;558:393–404. (6) Black RM, Clarke RJ, Read RW. Analysis of 1,1′-sulphonylbis[2-(methylsulphinyl)ethane] and 1-methylsulphinyl-2-[2-(methylthio)ethylsulphonyl]ethane, metabolites of sulphur mustard, in urine using gas chromatography-mass spectrometry. *J Chromatogr.* 1991;558:405–414. (7) Young CL, Ash D, Driskell WJ, et al. A rapid, sensitive method for the quantitation of specific metabolites of sulfur mustard in human urine using isotope-dilution gas chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2004;28:339–345. (8) Read RW, Black RM. Analysis of beta-lyase metabolites of sulfur mustard in urine by electrospray liquid chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2004;28:346–351. (9) Read RW, Black RM. Analysis of the sulfur mustard metabolite 1,1′-sulfonylbis[2-S-(N-acetylcysteinyl)ethane] in urine by negative ion electrospray liquid chromatography-tandem mass spectrometry. *J Anal Toxicol.* 2004;28:352–356. (10) Fidder A, Noort D, de Jong AL, Trap HC, de Jong LPA, Benschop HP. Monitoring of in vitro and in vivo exposure to sulfur mustard by GC/MS determination of the N-terminal valine adduct in hemoglobin after a modified Edman degradation. *Chem Res Toxicol.* 1996;9:788–792. (11) Noort D, Fidder A, Benschop HP, de Jong LP, Smith JR. Procedure for monitoring exposure to sulfur mustard based on modified Edman degradation of globin. *J Anal Toxicol.* 2004;28:311–315. (12) Black RM, Clarke RJ, Harrison JM, Read RW. Biological fate of sulphur mustard: identification of valine and histidine adducts in haemoglobin from casualties of sulphur mustard poisoning. *Xenobiotica.* 1997;27:499–512. (13) Noort D, Hulst AG, Trap HC, de Jong LPA, Benschop HP. Synthesis and mass spectrometric identification of the major amino acid adducts formed between sulphur mustard and haemoglobin in human blood. *Arch Toxicol.* 1997;71:171–178. (14) Noort D, Hulst AG, de Jong LP, Benschop HP. Alkylation of human serum albumin by sulfur mustard in vitro and in vivo: mass spectrometric analysis of a cysteine adduct as a sensitive biomarker of exposure. *Chem Res Toxicol.* 1999;12:715–721. (15) Noort D, Fidder A, Hulst AG, Woolfitt AR, Ash D, Barr JR. Retrospective detection of exposure to sulfur mustard: improvements on an assay for liquid chromatography-tandem mass spectrometry analysis of albumin-sulfur mustard adducts. *J Anal Toxicol.* 2004;28:333–338. (16) Capacio BR, Smith JR, DeLion MT, et al. Monitoring sulfur mustard exposure by gas chromatography-mass spectrometry analysis of thiodiglycol cleaved from blood proteins. *J Anal Toxicol*. 2004;28:306–310. (17) Van der Schans GP, Mars-Groenendijk R, de Jong LP, Benschop HP, Noort D. Standard operating procedure for immunoslotblot assay for analysis of DNA/sulfur mustard adducts in human blood and skin. *J Anal Toxicol.* 2004;28:316–319. (18) Noort D, Fidder A, Hulst AG, de Jong LP, Benschop HP. Diagnosis and dosimetry of exposure to sulfur mustard: development of a standard operating procedure for mass spectrometric analysis of haemoglobin adducts: exploratory research on albumin and keratin adducts. *J Appl Toxicol.* 2000;20(suppl 1):S187–S192.

were the first collected following hospital admission 5 to 10 days after the suspected exposure. Willems detailed the patients' medical histories.[101] Briefly, the injuries were described as moderate to severe and were consistent with sulfur mustard injury, including erythema and fluid-filled vesicles. Urine samples were initially analyzed for intact sulfur mustard, but were found to be negative. Following treatment of the urine samples with a strong acid to convert TDG to sulfur mustard, the samples were analyzed using GC-MS. The TDG concentrations found in the first set of samples collected at the hospital ranged between 5 to 100 ng/mL for the majority of the samples. The highest observed TDG concentration was 330 ng/mL from a casualty who died 1 day after admission. During the hospitalization, 18 to19 days after the exposure, a second set of urine samples was collected from one group. TDG levels in that set of samples were similar to observed background levels in control samples. TDG background levels were determined from urine samples obtained from nonexposed individuals and were generally less than 12 ng/mL, although two of the control samples had levels of 21 ng/mL and 55 ng/mL. Elevated background levels in control samples may indicate that this method also converts TDG-sulfoxide or some other analytes into sulfur mustard.

Drasch et al examined urine samples obtained during an autopsy of a sulfur mustard casualty for

unmetabolized sulfur mustard.[85] The victim was an Iranian soldier, age 24, who died of complications from pneumonia 7 days after the suspected exposure. The patient had been transferred to an intensive care unit in Munich, Germany. Samples were taken during the autopsy and stored at – 20°C for 1 year prior to analysis. Despite very high concentrations of sulfur mustard found in autopsy tissue specimens, sulfur mustard was not detected in the urine samples.

In 1990 Jakubowski et al received urine samples from an accidental laboratory exposure to sulfur mustard.[102] A liquid flashpoint tester overheated, vaporized a mixture that was thought to contain only a deconned solution, and exposed a chemist who had attempted to shut down the reaction. Nine hours after the incident, the individual felt a burning sensation on his arms, hands, neck, and face. Medical care was sought the morning after blisters appeared on his hands and arms. The erythematous and vesicated areas were estimated to be less than 5% and 1% of the total body surface area, respectively. The patient collected his total urine output for a 2-week period. For the first 3 days, the patient's total urine output was only about a third to half that of the average adult daily output of 1.5 liters, but the patient had a normal output level over the next 10 days. The assay method used measured both free and conjugated TDG.[87] The maximum TDG urinary excretion rate was 20 μg/day on the third day. TDG concentrations of 10 ng/mL or greater were observed in some samples for up to 1 week after the exposure. A rate constant was calculated for TDG concentration from days 4 through 10 and the half-life was found to be 1.2 days. A great deal of intraday variability was noted for the TDG urine concentrations. Consequently, the collection of several urine samples per day is recommended. An attempt was also made to estimate the total amount of sulfur mustard on the patient's skin. The estimate was based on two assumptions: 1) the assay for the free and conjugated TDG represents approximately 5% of the total amount of sulfur-mustard–related products in the blood, and 2) the bioavailability factor from skin to blood is 10 (ie, 10% of the sulfur mustard on the skin penetrated into the blood). A total of 0.243 mg of TDG was recovered over a 2-week period. This represents 4.86 mg in the blood, or 48.6 mg on the skin.

There are currently four instances of human exposure to sulfur mustard in which urine samples were subjected to several different assays in order to target multiple urinary metabolites. The first report described a small subset of urine samples previously analyzed by Wils et al[83] and were later reanalyzed by Black and Read[88] after storage at – 20°C for a 5-year period. Willems provided clinical information on the five individuals and coded them C1 through C5.[101] This group of individuals was reportedly exposed to exploding bombs that generated black dust and rain. Decontamination efforts consisted only of clothing removal, although some victims may have also showered. Early symptomatology included eye and throat irritation along with respiration difficulties. Within 1 to 2 days, victims developed erythema and small blisters on the skin, edema of the eyelids, photophobia, coughing, dyspnea, and hemoptysis. The patients were admitted to a hospital 10 days after the suspected sulfur mustard exposure. Urine samples were obtained at that time. Four of the five patients were discharged 26 to 37 days after hospitalization. Patient C1 developed adult respiratory distress syndrome and was given ventilatory support, but died in cardiovascular shock 15 days after the original exposure event (5 days after hospital admission). Wils et al coded the urine sample set from individuals C1 to C5 as G1 to G5 in their report and, using the GC-MS assay described above, found concentrations of TDG at 90 ng/mL, 45 ng/mL, 40 ng/mL, 40 ng/mL, and 15 ng/mL for individuals 1 through 5, respectively.[83] Using a different method measuring TDG and TDG-sulfoxide as a single analyte followed by GC-MS-MS analysis, Black and Read found TDG and TDG-sulfoxide levels of 69 ng/mL, 28 ng/mL, and 33 ng/mL for individuals C1, C2, and C5, respectively. C3 and C4 were not assayed because their samples were insufficient. Control urine samples analyzed by Black and Read produced a background level of 11 ng/mL.[88] Urine samples from all five casualties were analyzed for β-lyase concentrations, with the highest concentration found in patient C1. The concentrations found in the urine from four of the casualties ranged between 0.5 to 5 ng/mL, while the individual who died had a β-lyase concentration of 220 ng/mL.[88]

Once again using the GC-MS-MS method that measures both TDG and TDG-sulfoxide as a single analyte, Black and Read analyzed urine samples from two casualties of an alleged sulfur mustard attack on the Kurdish town of Halabja in 1988.[88] The patients had been transferred to London for medical treatment and urine samples were collected 13 days after the alleged incident. Black and Read found combined TDG plus TDG-sulfoxide levels of 11 ng/mL for both patients, but also found similar concentration levels in control samples. Urine samples were also analyzed for β-lyase concentrations using GC-MS-MS. Although the concentration of the β-lyase metabolites found in both patients was near the limit of detection for the assay, the analytes were clearly detectable and ranged between 0.1 and 0.3 ng/mL.[88] The urine samples were later analyzed using the LC-MS-MS assay that can distinguish the individual β-lyase metabolites.[96] The

monosulfoxide MSMTESE was only detected from one of the casualties and was near the limit of detection for the assay. The bissulfoxide SBMSE was detected in the urine from both casualties, but for each sample it was near the limit of detection of the assay (0.1–0.5 ng/mL).

Some of the most extensive testing of urine samples for sulfur-mustard–related metabolites involved two individuals who were accidentally exposed to a World War I munition containing sulfur mustard. The injuries were described as predominately cutaneous exposures, with both individuals exhibiting extensive skin blistering. Urine samples were collected 2 to 3 days after the individuals were exposed. Black and Read analyzed the urine using three different methods to detect metabolites of sulfur mustard hydrolysis.[91] In addition, the urine samples were examined for products of a reaction between sulfur mustard and GSH. The first assay measured TDG (free and conjugated together) and found concentrations of 2 ng/mL for each individual. The second assay targeted only free TDG-sulfoxide, and concentrations of 69 ng/mL and 45 ng/mL were found for the two individuals. Concentrations of 77 ng/mL and 54 ng/mL were found using a GC-MS-MS assay that measures TDG and TDG-sulfoxide as a single analyte. Control samples analyzed along with the patient samples for the second and third assays gave levels of 4 to 5 ng/mL, therefore the patient results were significantly higher than control values. The β-lyase metabolites were measured using both the GC-MS-MS method[91] and the LC-MS-MS method.[96] When the β-lyase metabolites were analyzed individually by LC-MS-MS, their concentrations ranged from 15 to 17 ng/mL and from 30 to 34 ng/mL for the monosulfoxide and bissulfoxide, respectively. When analyzed as the single, reduced form of SBMTE using GC-MS-MS, observed concentrations were 42 ng/mL and 56 ng/mL. Samples were also analyzed for a bis-(N-acetylcysteine) conjugate using LC-MS-MS.[97] This biomarker is also a reaction product of sulfur mustard and GSH. Although the biomarker is a major metabolite in rats exposed to sulfur mustard, it had not been reported in urine samples from exposed humans. The metabolite was found in urine samples from both exposed individuals, but concentrations were near the lower limit of detection (0.5–1 ng/mL).

The most recently reported exposure incident involved two explosive ordnance technicians who were part of a team tasked with destroying a suspected World War I 75-mm munition. The munition had been discovered in a clamshell driveway and was believed to have originated from material dredged from a seafloor dumping area. Following demolition procedures, two individuals came into contact with a brown oily liquid that was found leaking from the remnants of the munition. During the disposal operation, none of the ordnance team members complained of eye or throat irritation or breathing difficulties. The clinical sequence of events for one of the individuals has previously been reported,[103,104] but will be described in brief here. Within 2 hours of the munition destruction, one of the individuals (patient D1, a 35-year-old male) noticed a tingling sensation on one arm and then showered. The next morning (approximately 14 hours after the liquid contact), painful areas had developed on his hand, along with noticeable reddening and small blisters. He went to a local emergency room where the blisters were observed to grow and coalesce. He was subsequently transferred to a regional burn center. Blisters developed on the patient's arm, hand, ankle, and foot. The erythema and blistered area on the patient was estimated to be 6.5% of his body surface area (Figure 22-8). The patient never developed ocular or respiratory complications; consequently it appeared that his injuries were only the result of a cutaneous liquid exposure. The second individual (patient D2) had a small, single blister and was not hospitalized. Urine samples from patient D1 were collected on days 2 through 11 and 29, 35, and 42 days after exposure and from patient D2 on days 2, 4, and 7 after exposure. Reports containing preliminary analysis results mistakenly indicated that the first samples collected were 1 day post exposure.[104,105] Urine from both individuals was analyzed for hydrolysis metabolites and GSH reaction products.[104,105] Hydrolysis metabolites were determined using several different methods. Using a

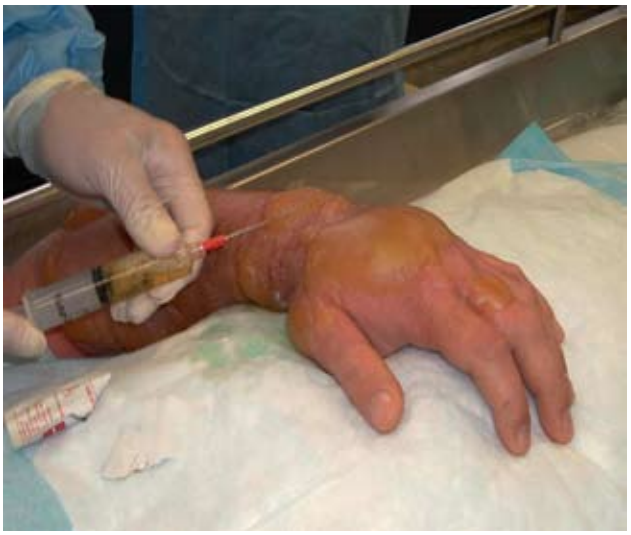


Fig. 22-8. Aspiration of blister fluid from accidental exposure to sulfur mustard.

GC-MS assay that targets free TDG and glucuronide-bound TDG,[87] detectable levels of TDG were only observed in patient D1's urine sample from day 2. Using a modified approach of the previous assay, the urine samples were incubated with concentrated HCl overnight to release acid-labile esters rather than submitted to an enzyme incubation step. TDG levels for patient D1 ranged from 40 ng/mL on day 2 to 10 ng/mL on day 6 after exposure using the modified assay.[104] TDG was not detected in urine samples beyond day 6 for patient D1 and was not detected in any of the urine samples from patient D2. Urine samples were also analyzed using a GC-MS-MS assay that measured both free and glucuronide-bound TDG.[105] There were no detectable levels in any of the urine samples from the individual with the single blister (patient D2). Patient D1 had the highest observed TDG concentration at day 2 (24 ng/mL). TDG concentrations ranged from 6 to 11 ng/mL over the next 5 days, decreased to a range of 1 to 2 ng/mL for the next 4 days, and TDG was undetected after day 11. The final method of analysis for hydrolysis metabolites was a GC-MS-MS assay that targeted free and glucuronide-bound TDG, free and glucuronide-bound TDG-sulfoxide, and acid-labile esters of TDG and TDG-sulfoxide.[105] The observed concentrations for patient D2 (2–4 ng/mL) fell within the range of concentrations previously observed in urine samples of unexposed individuals. The highest observed levels found in unexposed individuals with this assay were approximately 20 ng/mL.[89] While observed concentrations were much higher for patient D1, only days 2, 5, and 6 produced concentrations (50, 28, and 24 ng/mL, respectively) that were greater than the highest observed background control levels. β-lyase metabolites were measured as the single analyte SBMTE using GC-MS-MS.[105] Levels for both patients decreased dramatically by day 3 after exposure. Patient D2's urine SBMTE concentrations were 2.6 ng/mL, 0.8 ng/mL, and 0.08 ng/mL for samples taken 2, 4, and 7 days after exposure, respectively. Patient D1's concentrations decreased from 41 ng/mL at day 2 after exposure down to 7 ng/mL, 3.3 ng/mL, and 1.3 ng/mL over the next 3 days. For days 6 to 11, concentrations ranged between 0.07 and 0.02 ng/mL and SBMTE was not detected beyond day 11. Patient D1's urine from days 2 and 3 was also examined for the presence of the bis-(N-acetylcysteine) conjugate using LC-MS-MS. It was detected at a concentration of 3.1 ng/mL in the urine sample collected 2 days after exposure, but was not detected in the day 3 sample.

Currently the analytes of choice for assessing potential exposure to sulfur mustard in urine samples are the two β-lyase metabolites. The analytes can be measured individually using LC-MS-MS or reduced to a single analyte (SBMTE) and measured using GC-MS-MS. This has been verified in human exposure cases with sensitive and selective assays. To date, no known examples of background levels of these metabolites have been found in the urine from unexposed individuals (see Table 22-6, Table 22-10, Table 22-11).

Analysis of Blood Samples

Whereas urinary metabolites undergo relatively rapid elimination from the body, blood components offer biomarkers with potential use in verifying exposure to sulfur mustard long after the exposure incident. Three different approaches have been used for blood biomarker analysis. An intact macromolecule, such as protein or DNA, with the sulfur mustard adducts attached, can be analyzed. Currently, this approach has only been demonstrated for hemoglobin using in-vitro experiments. An alternate approach is to enzymatically digest the proteins to produce a smaller peptide with the sulfur mustard adduct still attached. Methods of this type have been developed for both hemoglobin and albumin. A third approach is to cleave the sulfur mustard adduct from the macromolecule and analyze it in a fashion similar to that used for free metabolites found in urine. The latter two approaches, described below, have both successfully verified human exposure to sulfur mustard.

Analytical Methods

Methods to measure sulfur mustard adducts to DNA in white blood cells have been developed using LC with fluorescence detection[106] and using an enzyme-linked immunosorbent assay (ELISA).[107,108] The DNA adduct that appears most abundant results from attachment of sulfur mustard to the N7 position of deoxyguaninosine (Figure 22-9a).[109] The immunochemical method used monoclonal antibodies that were raised against N7-(2-hydroxyethylthioethyl)-guanosine-5′-phosphate (Exhibit 22-2).

Hemoglobin is an abundant, long-lived protein in human blood. Alkylation reactions between sulfur mustard and hemoglobin have been shown to occur with six histidine, three glutamic acid, and two valine amino acids of hemoglobin.[110,111] Methods have been developed to analyze several of the adducts. While the histidine adducts appear to be the most abundant type, their analysis using MS techniques is problematic and the method does not appear to be as sensitive as the method for analyzing the N-terminal valine adducts.[112] Adducts to the N-terminal valine amino acids represent only a small fraction of the total alkylation of the macromolecule, but their location on the periphery of the molecule allows them to be selectively cleaved using a modified Edman degradation. Following

isolation of the globin from the RBCs, the globin is reacted with pentafluorophenyl isothiocyanate to form a thiohydantoin compound, which is further derivatized before analysis (Figure 22-10). The derivatized compound can then be analyzed using negative ion chemical ionization GC-MS (Exhibit 22-3).[113,114]

Human serum albumin was found to be alkylated by sulfur mustard at the cysteine-34 position. Fol-

TABLE 22-10

PUBLISHED REPORTS (1995–2006) OF LABORATORY ANALYSIS OF HUMAN URINE SAMPLES FOR HYDROLYSIS METABOLITES FOLLOWING SUSPECTED EXPOSURE TO SULFUR MUSTARD

Patient Sample Information*	Glucuronidase Incubation†	$TiCl_3$ Reduction‡	TDG-sulfoxide	Glucuronidase Incubation & $TiCl_3$ Reduction§
Iranian casualties, 3 of 5 individuals, treated at Ghent hospital, collected 10 days after incident (March 9, 1984)[1]	NM	Patient C1: 69 ng/mL Patient C2: 28 ng/mL Patient C5: 33 ng/mL Control: 11 ng/mL	NM	NM
Kurdish casualties, 2 individuals, treated at London hospital, collected 13 days after incident (March 17, 1988)[1]	NM	Patient L1: 11 ng/mL Patient L2: 11 ng/mL Control: 11 ng/mL	NM	NM
Accidental exposure to WWI munition, 2 individuals, collected 2–3 days after incident (1992)[2]	Patient S1: 2 ng/mL Patient S2: 2 ng/mL	Patient S1: 77 ng/mL Patient S2: 54 ng/mL Control: 4.5 ng/mL	Patient S1: 69 ng/mL Patient S2: 45 ng/mL Control: 5 ng/mL	NM
Accidental laboratory exposure, 1 individual, collected 2–14 days after incident (1990)[3]	Maximum excretion rate: 20 μg/day on day 3; concentration > 10 ng/mL for 1 week postexposure	NM	NM	NM
Accidental exposure to WWI munition, 2 individuals, collected 2–42 days after incident (July 19, 2004)[4]	Patient D1: 24, 9, 5, 14, 11, 6, 2, 2, 1.5, 1.2 ng/mL for days 2 to 11 after exposure, respectively	NM	NM	Patient D1: 50, 17, 11, 28, 24, 14, 4.5, 9, 5, 6 ng/mL for days 2 to 11 after exposure, respectively
	Patient D2: not detected days 2, 4, 7	NM	NM	Patient D2: 1.8, 3, 4.4 ng/mL for days 2, 4, 7, respectively

*These are the known details of the incident and sample collection time after suspected exposure.
†Assay measures TDG (free plus glucuronide-bound).
‡Assay measures free TDG, free TDG-sulfoxide, and acid-labile esters of both.
§Assay measures TDG (free plus bound), TDG-sulfoxide (free plus bound), and acid-labile esters of both.
NM: not measured
TDG: thiodiglycol
$TiCl_3$: titanium trichloride
WWI: World War I
Data sources: (1) Black RM, Read RW. Improved methodology for the detection and quantitation of urinary metabolites of sulphur mustard using gas chromatography-tandem mass spectrometry. *J Chromatogr B Biomed Appl*. 1995;665:97–105. (2) Black RM, Read RW. Biological fate of sulphur mustard, 1,1′-thiobis(2-chloroethane): identification of beta-lyase metabolites and hydrolysis products in human urine. *Xenobiotica*. 1995;25:167–173. (3) Jakubowski EM, Sidell FR, Evans RA, et al. Quantification of thiodiglycol in human urine after an accidental sulfur mustard exposure. *Toxicol Methods*. 2000;10:143–150. (4) Barr JR, Young CL, Woolfit AR, et al. Comprehensive quantitative tandem MS analysis of urinary metabolites and albumin adducts following an accidental human exposure to sulfur mustard. In: *Proceedings of the 53rd Conference of the American Society of Mass Spectrometry*. San Antonio, Tex: June 5–9, 2005.

TABLE 22-11

PUBLISHED REPORTS (1995–2006) OF LABORATORY ANALYSIS OF HUMAN URINE SAMPLES FOR GLUTATHIONE REACTION PRODUCTS FOLLOWING A SUSPECTED EXPOSURE TO SULFUR MUSTARD

Patient Sample Information*	β-lyase Metabolites†	β-lyase Metabolites‡	Bis-(N-acetylcysteine) Conjugate§
Iranian casualties, 5 of 5 individuals. treated at Ghent hospital, collected 10 days after incident (March 9, 1984)[1]	Patient C1: 220 ng/mL Patient C2: 0.5 ng/mL Patient C3: 1 ng/mL Patient C4: 5 ng/mL Patient C5: 1 ng/mL	NM	NM
Kurdish casualties, 2 individuals, treated at London hospital, collected 13 days after incident (March 17, 1988)[1,2]	Patient L1: 0.1 ng/mL Patient L2: 0.3 ng/mL	Patient L1: MSMTESE = <0.1 ng/mL, SBMSE = ~ 0.1 ng/mL Patient L2: MSMTESE = 0.1 ng/mL, SBMSE = ~ 0.2 ng/mL	NM
Accidental exposure to WWI munition, 2 individuals, collected 2–3 days after incident (1992)[2,3,4]	Patient S1: 42 ng/mL Patient S2: 56 ng/mL	Patient S1: MSMTESE = 15 ng/mL, SBMSE = 30 ng/mL Patient S2: MSMTESE = 17 ng/mL, SBMSE = 34 ng/mL	Patient S1: 1 ng/mL Patient S2: 1 ng/mL
Accidental exposure to WWI munition, 2 individuals, collected 2–42 days after incident (July 19, 2004)[5]	Patient D1: 41, 7, 3.3, 1.3 ng/mL for days 2–5 after exposure, respectively; 0.07–0.02 ng/mL for days 6–11 after exposure Patient D2: 2.6, 0.8, 0.08 ng/mL for days 2, 4, 7, respectively	NM	Patient D1: 3.1 ng/mL

*This information includes known incident information and sample collection time after suspected exposure.
†Using GC-MS-MS analysis
‡Using LC-MS-MS analysis
§Using LC-MS-MS analysis
GC: gas chromatography
LC: liquid chromatography
MS: mass spectrometry
MSMTESE: 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane
NM: not measured
SBMSE: 1,1′-sulfonylbis[2-(methylsulfinyl)ethane]
Data sources: (1) Black RM, Read RW. Improved methodology for the detection and quantitation of urinary metabolites of sulphur mustard using gas chromatography-tandem mass spectrometry. *J Chromatogr B Biomed Appl*. 1995;665:97–105. (2) Read RW, Black RM. Analysis of beta-lyase metabolites of sulfur mustard in urine by electrospray liquid chromatography-tandem mass spectrometry. *J Anal Toxicol*. 2004;28:346–351. (3) Black RM, Read RW. Biological fate of sulphur mustard, 1,1′-thiobis(2-chloroethane): identification of beta-lyase metabolites and hydrolysis products in human urine. *Xenobiotica*. 1995;25:167–173. (4) Read RW, Black RM. Analysis of the sulfur mustard metabolite 1,1′-sulfonylbis[2-S-(N-acetylcysteinyl)ethane] in urine by negative ion electrospray liquid chromatography-tandem mass spectrometry. *J Anal Toxicol*. 2004;28:352–356. (5) Barr JR, Young CL, Woolfit AR, et al. Comprehensive quantitative tandem MS analysis of urinary metabolites and albumin adducts following an accidental human exposure to sulfur mustard. In: *Proceedings of the 53rd Conference of the American Society of Mass Spectrometry*. San Antonio, Tex: June 5–9, 2005.

lowing isolation of the albumin from the blood, the albumin can be reacted with pronase enzyme to digest protein. One of the resulting peptide fragments is a tripeptide of the sequence cysteine-proline-phenylalanine, which contains the sulfur-mustard–alkylated cysteine-34. After solid phase extraction, the tripeptide was analyzed using LC-MS-MS.[115] The lower limit of detection for the assay (1 nM) was once again reported as an equivalent exposure level, as determined using in-vitro exposures to sulfur mustard in human whole blood. Recently, a modification to the isolation of the albumin from blood was reported using affinity chro-

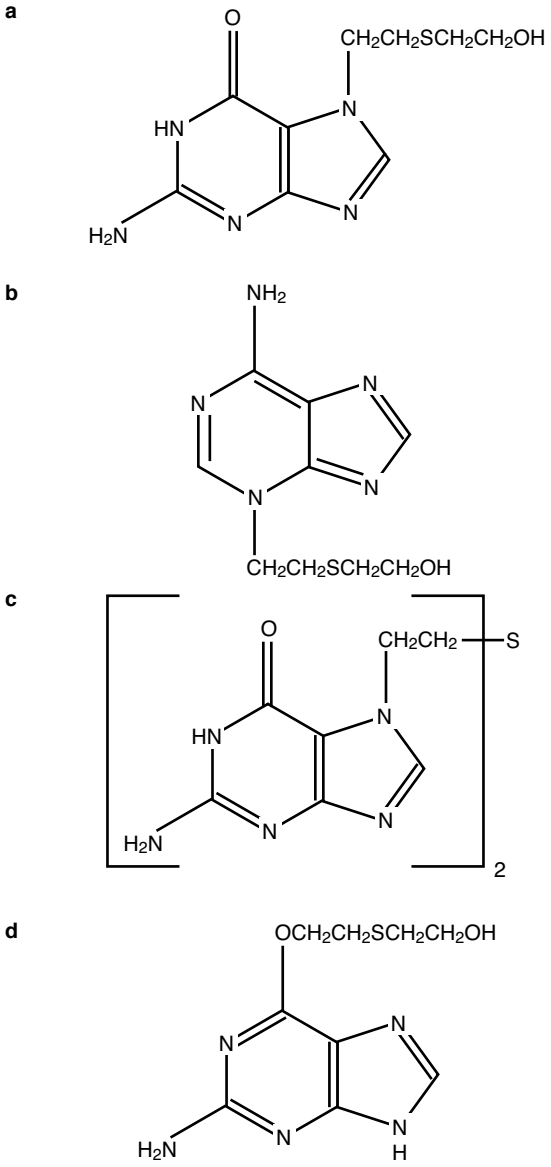


Fig. 22-9. Deoxyribonucleic acid adducts resulting from reaction with sulfur mustard. **(a)** N7-HETE-guanine. **(b)** N3-HETE-adenine. **(c)** Bis[2-(guanin-7-yl)ethyl]sulfide. **(d)** O^6-HETE-guanine.

matography rather than the precipitation procedure.[116] The modified procedure significantly reduced the sample preparation time.

The final method for analyzing blood samples to be discussed targets blood proteins in a more general approach. It was previously shown that sulfur mustard adducts of glutamic and aspartic acids to keratin could be cleaved using base.[117] Using a similar approach, precipitated proteins from plasma, whole blood, or RBCs were treated with base to liberate the sulfur mustard adduct, hydroxyethylthioethyl, from the protein. Upon release, the adduct (in the form of TDG) was derivatized and analyzed using negative-ion chemical ionization GC-MS (Figure 22-11; see Exhibit 22-3). The lower limit of detection for the assay in plasma was 25 nM, as determined using in-vitro exposures of sulfur mustard in human plasma.[118]

The limits of detection or equivalent levels of exposure to sulfur mustard reported for most of the blood assays are based on in-vitro exposures of whole blood or plasma. Quantitation of patient samples report the amount of sulfur mustard adducts that are found in the samples relative to the amount of adducts that are found from in-vitro exposures of whole blood or plasma at various known concentrations of sulfur mustard. Choosing whole blood over plasma to generate the in-vitro standard curves produces very different results because sulfur mustard readily reacts with hemoglobin. Additionally, the technique used for generating in-vitro standards can have significant effects. For example, approximately a 30% difference was observed for the generation of two in-vitro standard curves, depending on how the sulfur mustard was incubated in blood.[119] Higher adduct levels were observed when the sulfur mustard was allowed to react with the blood for 2 hours at 37°C, as opposed to 4 hours at room temperature.

Application to Human Exposure

Blood samples following a suspected human exposure to sulfur mustard rarely become available for laboratory analysis. Three of the five known reports involve the analysis of samples that were taken from casualties of the Iran-Iraq War, frozen for several years, then reanalyzed to verify exposure as new methods were developed. The other two published reports are on the analysis of blood samples obtained from three individuals who were casualties of accidental exposures to World War I munitions.

The blood from two Iranian casualties who were believed to have been exposed to sulfur mustard in 1988 was analyzed using both the ELISA method for DNA adducts and the GC-MS method for the analysis of the N-terminal valine of hemoglobin.[120] Samples were collected 22 and 26 days following the suspected exposure. One of the casualties had skin injuries that were consistent with an exposure to sulfur mustard, but the second casualty had injuries that were described as only "vaguely compatible" with sulfur mustard exposure. Both individuals had approximately the same level of hemoglobin valine adduct, equivalent to the amount observed from a 900-nM, in-vitro, sulfur mustard exposure in whole blood. ELISA DNA adduct levels observed in the granulocytes were also similar

EXHIBIT 22-2

SAMPLE PREPARATION PROCEDURE FOR SULFUR MUSTARD ADDUCTS TO DEOXYRIBONUCLEIC ACID IN BLOOD

Procedure of van der Schans et al for immunuslotblot analysis:

- Collect blood specimen in vacutube containing EDTA.
- Isolate and denature DNA using following procedure:
  - Transfer 0.3 mL of blood to Eppendorf tube.
  - Add RBC lysis solution, mix, and centrifuge.
  - Lyse pelleted WBCs with cell lysis solution containing proteinase K.
  - Shake solution for 1 hour.
  - Treat solution with RNAse.
  - Add protein precipitation solution, mix, and centrifuge.
  - Transfer supernatant to tube containing isopropanol and centrifuge.
  - Wash pellet with 70% ethanol, centrifuge, and air dry.
  - Dissolve pellet overnight in Tris buffer containing HCl and EDTA.
  - Determine DNA concentration using UV-VIS spectrometer.
  - Prepare DNA solution with formamide, formaldehyde, and Tris buffer.
  - Incubate solution at 52°C.
  - Cool rapidly on ice and store at – 20°C.
- Immunuslotblot assay procedure:
  - Dilute denatured DNA samples in PBS.
  - Spot DNA solution onto nitrocellulose filter.
  - Wash spotted sample with PBS and air-dry filter.
  - Cross-link DNA to filter using UV-gene-cross-linker.
  - Incubate filter with blocking solution.
  - Wash with PBS/Tween solution.
  - Incubate filter overnight with monoclonal antibodies that recognize N7-(2-hydroxyethylthioethyl)-2′-deoxyguanosine at 4°C with continuous shaking.
  - Wash with PBS/Tween solution.
  - Incubate filter with rabbit-anti-mouse-Ig-horseradish peroxidase antibody for 2 hours at room temperature with shaking.
  - Wash with PBS/Tween solution.
  - Blot dry filter and transfer into a luminometer cassette.
  - Measure luminescence.

DNA: deoxyribonucleic acid
EDTA: ethylenediaminetetraacetic acid
HCl: hydrochloric acid
Ig: immunoglobulin
PBS: phosphate-buffered saline
RBC: red blood cell
RNAse: ribonuclease
Tris: trishydroxymethylaminomethane
UV-VIS: ultraviolet-visible spectrophotometry
WBC: white blood cell
Data source: (1) Van der Schans GP, Mars-Groenendijk R, de Jong LP, Benschop HP, Noort D. Standard operating procedure for immunoslotblot assay for analysis of DNA/sulfur mustard adducts in human blood and skin. *J Anal Toxicol.* 2004;28:316–319.

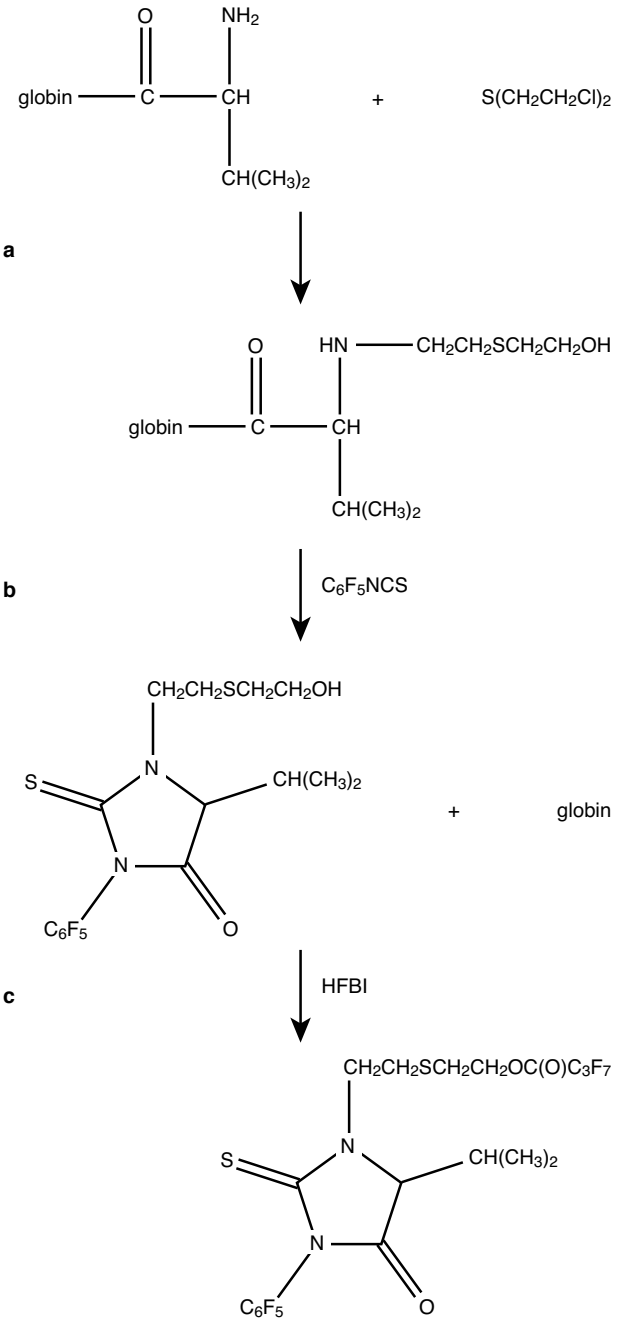


Fig. 22-10. Analytical method of Fidder et al (1996) for blood exposed to sulfur mustard. **(a)** Reaction of N-terminal valine of globin with sulfur mustard. **(b)** Modified Edman degradation of N-terminal valine-sulfur mustard adduct. **(c)** Derivatization using HFBI.
HFBI: heptafluorobutyryl imidazole
Data source: Fidder A, Noort D, de Jong AL, Trap HC, de Jong LPA, Benschop HP. Monitoring of in vitro and in vivo exposure to sulfur mustard by GC/MS determination of the N-terminal valine adduct in hemoglobin after a modified Edman degradation. *Chem Res Toxicol.* 1996;9:788–792.

for both individuals, 150 to 160 nM. The individual with the skin injuries consistent with sulfur mustard exposure had observed ELISA DNA adduct levels in the lymphocytes that were only about half that observed in the individual with injuries that were less pronounced: 220 nM and 430 nM, respectively.

Blood samples obtained in 1986 from a group of Iranian casualties that were treated at a hospital in Ghent for injuries believed to have been caused by sulfur mustard were examined years later using MS methods by Black et al for both valine and histidine adducts of hemoglobin.[114] The four individuals had blood samples collected at 5 or 10 days following the suspected exposure event. Levels of the valine adduct ranged between 0.3 and 0.8 ng/mL. Observed levels of the histidine adduct were greater than the amount of valine adduct and ranged between 0.7 and 2.5 ng/mL. Using the same methods, Black et al also examined blood from one of the two individuals who were accidentally exposed to a World War I sulfur mustard munition.[114] Several urinary metabolites were detected in specimens from this individual, indicating exposure to sulfur mustard (see above). In a blood sample obtained 2 days after the exposure, the valine and histidine hemoglobin adduct levels were 0.3 ng/mL and 2.5 ng/mL, respectively.

Blood samples obtained from nine Iranian casualties of sulfur mustard exposure were analyzed for the N-terminal valine adduct of hemoglobin using GC-MS[121] and for the albumin cysteine adduct using LC-MS-MS.[115] All nine individuals were hospitalized and had skin changes consistent with sulfur mustard exposure. Several of the casualties also had respiratory difficulties. Blood samples were collected between 8 and 9 days after the exposure incident. Exposure levels of the patient blood samples were correlated with human whole blood that was exposed to sulfur mustard in vitro. Adduct levels for both the hemoglobin valine adduct and for the albumin cysteine adduct were in very close agreement with each other. Observed exposure levels were between 0.3 and 2 μm and 0.4 and 1.8 μm for the hemoglobin and albumin adducts, respectively.

The final exposure incident to be discussed involved the two individuals who were accidentally exposed to a World War I munition in 2004. Details of the exposure were given earlier in the urine section. This particular human exposure to sulfur mustard differed from nearly all other previously reported incidents in several important aspects. The two individuals are only the second and third casualties of sulfur mustard exposure to have both urine and blood samples made available for laboratory testing. Generally, urine or

EXHIBIT 22-3

SAMPLE PREPARATION METHODS FOR THE GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC ANALYSIS OF SULFUR MUSTARD ADDUCTS TO BLOOD BIOMOLECULES

Procedure of Fidder et al for sulfur mustard adduct to N-terminal valine of hemoglobin*:

- Isolate globin from blood using following procedure:
  - Centrifuge blood and remove plasma layer.
  - Wash RBCs with saline solution.
  - Lyse RBCs with water and place solution into ice water bath.
  - Centrifuge and transfer supernatant into tube containing HCl/acetone.
  - Wash the precipitate with HCl/acetone, acetone, and ether.
  - Dry precipitate.
- Mix isolated globin from blood sample with internal standard (globin isolated from blood that was previously exposed to deuterated sulfur mustard).
- Dissolve globin in formamide.
- Add pyridine and pentafluorophenyl isothiocyanate to solution.
- Incubate solution at 60°C for 2 hours.
- Add toluene, mix, centrifuge, and freeze samples in liquid nitrogen.
- Remove toluene layer and wash with water, aqueous Na_2CO_3, and water.
- Dry toluene with $MgSO_4$, evaporate to dryness, and dissolve in toluene.
- Filter solution through a preconditioned Florisil solid phase extraction cartridge.
- Wash cartridge with dichloromethane.
- Elute with methanol/dichloromethane.
- Evaporate to dryness and dissolve in toluene.
- Add heptafluorobutyryl imidazole and heat solution.
- After cooling, wash with water, aqueous Na_2CO_3, and water.
- Dry toluene with $MgSO_4$ and concentrate solution.
- Analyze using GC-MS with methane negative ion chemical ionization.[1]

Procedure of Capacio et al for sulfur mustard adducts to aspartic and glutamic acid residues of blood proteins:

- Precipitate blood proteins:
  - For plasma samples, use acetone.
  - For whole blood or RBCs, use HCl/acetone.
- Centrifuge solution and discard supernatant.
- Wash protein pellet with acetone and ether.
- Centrifuge solution and discard supernatant.
- Dry protein at room temperature.
- Add dried protein to NaOH solution.
- Heat solution at 70°C for 1.5 hours.
- Neutralize solution with HCl and dry with sodium sulfate.
- Add ethyl acetate, mix, and remove ethyl acetate layer.
- Add internal standard (deuterated thiodiglycol) to ethyl acetate and dry with sodium sulfate.
- Add pyridine and pentafluorobenzoyl chloride.
- After 10 minutes, add water and sodium bicarbonate.
- Remove ethyl acetate layer and dry with sodium sulfate.
- Pass ethyl acetate through a preconditioned silica solid-phase–extraction cartridge and collect the filtered solution.
- Pass additional ethyl acetate through cartridge, collect, and combine the two fractions.
- Analyze using GC-MS with methane negative ion chemical ionization.[2]

(**Exhibit 22-3** *continues*)

Exhibit 22-3 *continued*

*The lower limit of detection for the assay was determined using in vitro exposures of sulfur mustard in human whole blood and was determined to be equivalent to a 100 nM exposure level.[1,3] Following the administration of a single dose of sulfur mustard to a marmoset (4.1 mg/kg; intravenous), the valine adduct was still detected in blood taken 94 days later.[4] Intact hemoglobin with the sulfur mustard adducts attached have been examined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, but to date the technique has only been utilized for in vitro experiments at relatively high concentrations of sulfur mustard.[5]
GC: gas chromatography
HCl: hydrochloric acid
MS: mass spectrometry
RBC: red blood cell
Data sources: (1) Fidder A, Noort D, de Jong AL, Trap HC, de Jong LPA, Benschop HP. Monitoring of in vitro and in vivo exposure to sulfur mustard by GC/MS determination of the N-terminal valine adduct in hemoglobin after a modified Edman degradation. *Chem Res Toxicol*. 1996;9:788–792. (2) Capacio BR, Smith JR, DeLion MT, et al. Monitoring sulfur mustard exposure by gas chromatography-mass spectrometry analysis of thiodiglycol cleaved from blood proteins. *J Anal Toxicol*. 2004;28:306–310. (3) Noort D, Fidder A, Benschop HP, de Jong LP, Smith JR. Procedure for monitoring exposure to sulfur mustard based on modified edman degradation of globin. *J Anal Toxicol*. 2004;28:311–315. (4) Noort D, Benschop HP, Black RM. Biomonitoring of exposure to chemical warfare agents: a review. *Tox Appl Pharmacol*. 2002;184:116–126. (5) Price EO, Smith JR, Clark CR, Schlager JJ, Shih ML. MALDI-ToF/MS as a diagnostic tool for the confirmation sulfur mustard exposure. *J Appl Toxicol.* 2000;20(suppl 1):S193–S197.

blood samples that are collected and made available for verifying sulfur mustard exposure are from a single time point after the exposure. In this instance, the patient with more severe injuries (patient D1, see above) had blood and urine collected almost daily for the first 10 days after exposure and then again on days 29, 35, and 42. The incident also provided an opportunity to examine blood and urine metabolite levels from individuals with very different levels of injury. Patient D1 had extensive vesication of the arm and leg, while patient D2 developed only a single small blister. (The urinary metabolite results were detailed earlier in this section.) As expected, the observed concentrations of both urine hydrolysis metabolites and GSH reaction products were much greater in patient D1. Sulfur mustard metabolite concentrations in the blood were also much greater in patient D1. Blood metabolites were assayed using two different methods. The first assay targeted the sulfur mustard adduct to cysteine-34 of albumin using pronase digestion of the protein followed by LC-MS-MS analysis.[116] Based on in-vitro exposures to sulfur mustard in human whole blood, concentrations of albumin adducts found in the plasma of patient D1 were 350 nM on day 2 after the exposure and had decreased by 74% (90 nM) on day 42.[105] The rate of decrease over that time was consistent with the reported half-life of human albumin of 21 days. Albumin adduct concentrations for patient D2 over the sample collection period of 2 to 7 days after exposure remained stable and ranged between 16 and 18 nM. The second assay targeted plasma protein adducts by cleaving the adduct with base, followed by analyzing the derivatized adduct using negative ion chemical ionization GC-MS.[118] This was the first reported use of this assay in the verification of a human exposure to sulfur mustard. Concentrations of the plasma protein adducts were 97 nM on day 2 and decreased by 76% (23 nM) by day 42, based on in-vitro exposures to sulfur mustard in human plasma instead of whole blood.[122] The assay could not detect plasma protein adducts in patient D2. The assay was modified slightly to lower the reported lower limit of detection of 25 nM, but the limited amount of plasma received did not permit

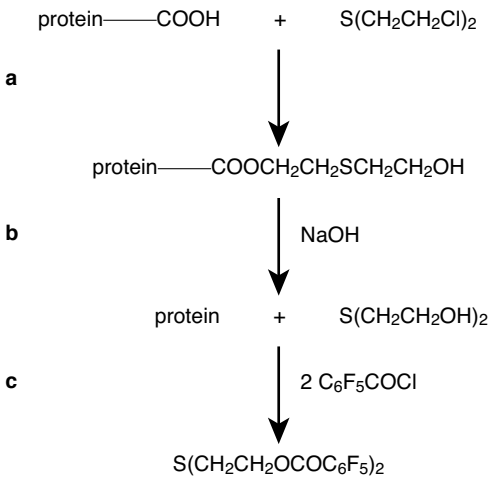


Fig. 22-11. Analytical approach of Capacio et al. **(a)** Reaction of protein carboxylic acid groups with sulfur mustard. **(b)** Hydrolysis of the ester groups to release thiodiglycol. **(c)** Derivatization of thiodiglycol using pentafluorobenzoyl chloride.
Data source: Capacio BR, Smith JR, DeLion MT, et al. Monitoring sulfur mustard exposure by gas chromatography-mass spectrometry analysis of thiodiglycol cleaved from blood proteins. *J Anal Toxicol.* 2004;28:306–310.

reanalysis for patient D2 using the modified method.

Blood provides several options for assessing potential exposure to sulfur mustard because a variety of different metabolites have been verified in human exposure cases. Most of the assays are sensitive and selective, and the majority of the methods use gas or LC combined with MS. Background levels have not been found in the blood from unexposed individuals. The time period between exposure and sample collection and the severity of the injury are probably the most important factors to consider when selecting the appropriate assay. Currently the most sensitive assay targets the alkylated cysteine of albumin, but alkylated hemoglobin should offer a biomarker of greater longevity (Table 22-12).

Analysis of Other Biomedical Sample Types (Tissue, Hair, Skin, Blister Fluid)

Urine and blood have been the traditional biomedical samples used to test for exposure to sulfur mustard; there are few reports on the analysis of other types of biomedical samples. Using the same method described in the urine methods section, Drasch et al analyzed several tissue types obtained from an autopsy for unmetabolized sulfur mustard.[85] An Iranian soldier, age 24, died of complications of pneumonia 7 days after a suspected exposure to sulfur mustard. The patient had been transferred to an intensive care unit in Munich, Germany for treatment. Tissue samples were taken during the autopsy, stored at –20°C for 1 year, and then analyzed for unmetabolized sulfur mustard. Sulfur mustard was found in the highest concentrations in the victim's fat, skin, brain, and kidney; concentrations ranged from 5 to 15 mg/kg. Lesser amounts were found in the patient's muscle, liver, spleen, and lung and ranged from approximately 1 to 2 mg/kg.[85]

Hair specimens have been analyzed for unmetabolized sulfur mustard using a methylene chloride extraction followed by analysis with GC-MS.[123] Hair samples that were obtained from two casualties of the Iran-Iraq War in 1986 were analyzed for unmetabolized sulfur mustard.[123] The two casualties were examined by a United Nations inspection team as part of an "alleged use" investigation initiated at Iran's request. The clinical history of the patients was consistent with sulfur mustard exposure. Additionally, analysis of vapor and soil samples from the bomb site tested positive for sulfur mustard. The inspection team was presented with hair samples from two individuals that were reportedly exposed the previous day. The hair specimens were sent to the National Defence Research Institute of Sweden for analysis. One of the hair samples produced a positive response for sulfur mustard estimated to be between 0.5 and 1.0 μg/g. The second patient's hair sample tested negative for sulfur mustard.[123]

Using the urine testing method to measure TDG levels, Wils et al assayed pieces of skin that were taken from sulfur mustard casualties treated in the Ghent hospital. They found concentrations of TDG in the range of 2 to 7 $\mu g/g$ in the skin samples.[84] Immunochemical detection assays for sulfur mustard adducts in human skin have been developed for keratin[124] and DNA.[108] To date, the assays have not been used on skin samples following a suspected human exposure to sulfur mustard.

Fluid removed from blisters resulting from sulfur mustard exposure has also been analyzed from two casualties. Jakubowski et al obtained a small amount of blister fluid from an accidental laboratory exposure that was detailed in the urine section above. The blister fluid was injected directly into a GC-MS for analysis. Neither unmetabolized sulfur mustard nor TDG were observed in the blister fluid, but a polymer of TDG appeared to be present.[102] The most recently reported exposure also generated blister fluid samples that were made available for analysis (see Figure 22-8). Korte et al examined blister fluid that was obtained on days 2 and 7 after exposure for both free TDG and protein-bound adducts of sulfur mustard.[122] Free TDG concentrations were 19 ng/mL and 24 ng/mL for days 2 and 7, respectively. The protein-bound adducts were measured using the same method used for plasma protein adducts.[118] The observed levels were 63 and 73 pg/mg of protein for days 2 and 7, respectively. Blister fluid from day 7 was also assayed with the albumin tripeptide LC-MS-MS method[116] previously used for plasma samples from the same individual. The concentration of the alkylated tripeptide reported using this assay was similar to the plasma concentration found for the same day.[105]

Since 1995 there has been a significant increase in the reported number of laboratory methods for verifying human exposure to sulfur mustard. Sensitivity to the analytical methods continues to improve and a number of new biomarkers have been identified and verified in biomedical samples from exposed individuals. These advances have helped verify even low doses of sulfur mustard exposure and have extended the time period from exposure event to collection. The major drawback to the current laboratory methodologies is that they require expensive instrumentation, highly trained personnel, and analytical standards that are generally not commercially available. Consequently, the time interval from sample collection, transport to an appropriate laboratory, sample preparation, instrument configuration,

TABLE 22-12

PUBLISHED REPORTS (1997–2006) OF LABORATORY ANALYSIS OF HUMAN BLOOD SAMPLES FOLLOWING SUSPECTED EXPOSURE TO SULFUR MUSTARD

Patient Sample Information*	DNA Adduct†	Hemoglobin Adduct‡	Hemoglobin Adduct§	Albumin Adduct¥	Blood Protein Adducts¶
Iranian casualties, 2 individuals, collected 22 days (Patient 1) & 26 days (Patient 2) after incident (1988)[1]	Patient 1: lymphocytes = 220 nM, granulocytes = 160 nM Patient 2: lymphocytes = 430 nM, granulocytes = 150 nM	Patient 1: 900 nM Patient 2: 900 nM	NM	NM	NM
Iranian casualties, 4 individuals, treated at Ghent hospital; collected 5 & 10 days after incident (1986)[2]	NM	Range: 0.3–0.8 ng/mL	Range: 0.7–2.5 ng/mL	NM	NM
Accidental exposure to WWI munition; 1 individual, collected 2 days after incident (1992)[2]	NM	0.3 ng/mL	2.5 ng/mL	NM	NM
Iranian casualties, 9 individuals, treated at Utrecht hospital; collected 8–9 days after incident (1986)[3,4]	NM	Range: 0.3–2 μM	NM	Range: 0.4–1.8 μM	NM
Accidental exposure to WWI munition; 2 individuals, collected 2–42 days after incident (2004)[5,6]	NM	NM	NM	Patient D1: 350 and 90 nM for days 2 & 42 after exposure respectively; Patient D2: 16–18 nM for days 2, 4, 7 after exposure	Patient D1: 97 and 23 nM for days 2 & 42 after exposure, respectively

*This information includes known incident information and sample collection time after suspected exposure.
†N7-(2-HETE)-2′-deoxyguanosine
‡(HETE)-N-terminal valine
§(HETE)-histidine
¥S-[2-(HETE)]-Cys-Pro-Phe
¶(HETE)-aspartic & glutamic acids
DNA: deoxyribonucleic acid
HETE: hydroxyethylthioethyl
NM: not measured

Data sources: (1) Benschop HP, van der Schans GP, Noort D, Fidder A, Mars-Groenendijk RH, de Jong LP. Verification of exposure to sulfur mustard in two casualties of the Iran-Iraq conflict. *J Anal Toxicol.* 1997;21:249–251. (2) Black RM, Clarke RJ, Harrison JM, Read RW. Biological fate of sulphur mustard: identification of valine and histidine adducts in haemoglobin from casualties of sulphur mustard poisoning. *Xenobiotica*. 1997;27:499–512. (3) Noort D, Hulst AG, de Jong LP, Benschop HP. Alkylation of human serum albumin by sulfur mustard in vitro and in vivo: mass spectrometric analysis of a cysteine adduct as a sensitive biomarker of exposure. *Chem Res Toxicol.* 1999;12:715–721. (4) Benschop HP, Noort D, van der Schans GP, de Jong LP. Diagnosis and dosimetry of exposure to sulfur mustard: development of standard operating procedures; further exploratory research on protein adducts. Rijswijk, The Netherlands: TNO Prins Maurits Laboratory. Final report DAMD17-97-2-7002, ADA381035, 2000. (5) Barr JR, Young CL, Woolfit AR, et al. Comprehensive quantitative tandem MS analysis of urinary metabolites and albumin adducts following an accidental human exposure to sulfur mustard. In: *Proceedings of the 53rd Conference of the American Society of Mass Spectrometry.* San Antonio, Tex: June 5–9, 2005. (6) Korte WD, Walker EM, Smith JR, et al. The determination of sulfur mustard exposure by analysis of blood protein adducts. *Wehrmed Mschr*. 2005;49:327.

and finally an analytical result is long. Because exposure to sulfur mustard can occur without overt or immediate clinical signs and the onset of symptoms can be delayed for many hours, there is interest in developing diagnostic methods for the field and patient care areas. Ongoing research in the use of immunochemical assays is one area in particular that offers hope for a field-forward assay.

LEWISITE

Background

Lewisite is an arsenical vesicant with a small molecular weight primarily found in the trans isomeric form, although it also exists in cis and geminal forms.[125] Stockpiles of lewisite or lewisite mixed with sulfur mustard reportedly exist in a number of countries and present a potential risk for accidental exposure. Most analytical methods that have been reported in the public scientific literature regarding lewisite or related compounds are for the sample preparation and analysis of environmental samples. In the past 10 years, there have only been a handful of reports regarding the analysis of biomedical samples to confirm lewisite exposure. Much like the other chemical warfare agents, lewisite is readily hydrolyzed in aqueous solutions, including biological fluids. Therefore, the likelihood of finding the parent compound in a biomedical sample, such as blood or urine, is minimal. Consequently, method development has focused on the breakdown compounds of lewisite or on products formed from its interaction with biomolecules. Until recently, most assays for lewisite have involved analyzing elemental arsenic using techniques such as atomic absorption spectroscopy. A drawback of this approach is that it lacks specificity because arsenic is ubiquitous in the environment. In addition to naturally occurring sources, arsenic is also found in some commercial products and food items (particularly marine organisms). Arsenic is also a by-product of several industrial processes.

Analysis of Urine and Blood Samples

Lewisite rapidly reacts with water to form chlorovinylarsonous acid (CVAA). CVAA slowly converts to the arsinoxide form and polymerization reactions can occur. Earlier studies indicated that animals (species not specified) exposed to lewisite either topically or via injection were found to have measurable levels of CVAA in their urine and throughout their digestive systems.[126]

Specific biomarkers of lewisite exposure are currently based on a limited number of in-vitro[127,128] and animal studies.[129,130] Wooten et al developed a solid phase microextraction headspace sampling method for urine samples, followed by GC-MS analysis.[128] It is the most sensitive method reported to date, with a lower limit of detection of 7.4 pg/mL. Animal experiments have been limited in number and scope. In one study of four animals, guinea pigs were given a subcutaneous dose of lewisite (0.5 mg/kg). Urine samples were analyzed for CVAA using both GC-MS and GC coupled with an atomic emission spectrometer set for elemental arsenic.[129] The excretion profile indicated a very rapid elimination of CVAA in the urine. The mean concentrations detected were 3.5 μg/mL, 250 ng/mL, and 50 ng/mL for the 0- to 8-hour, 8- to 16-hour, and 16- to 24-hour samples, respectively. Trace level concentrations (0–10 ng/mL) of CVAA were detected in the urine of the 24- to 32-hour and 32- to 40-hour samples. The second animal study also used a subcutaneous dose of lewisite (0.25 mg/kg) given to four guinea pigs.[130] Using GC-MS, CVAA was detected in urine samples up to 12 hours following exposure. In the same experiment, blood from the animals was also analyzed using GC-MS to detect CVAA. The amount of measured CVAA was the sum of CVAA that was displaced from hemoglobin along with free CVAA in the blood. The assay was able to detect the analyte at 10 days after the exposure, although the concentration was only 10% of that found 24 hours after exposure. Following the incubation of human blood with radiolabeled lewisite, Fidder et al found that 90% of the radioactivity was associated with the RBCs, and 25% to 50% was found with the globin.[130] Because of CVAA's reactive nature, derivatization using a thiol compound has generally been applied as part of the sample preparation process (Figure 22-12, Exhibit 22-4).

Application to Human Exposure

There are currently no reports of the collection of biomedical samples from individuals with suspected lewisite exposure. Samples from such incidents are critical to confirm the validity of assaying for the biomarkers observed in animal models. Additionally, the biomarkers that have been investigated in animal studies have indicated a rapid clearance of those biomarkers in urine and less so for blood.[130] This creates problems for the retrospective determination of lewisite exposure beyond a few days when analyzing

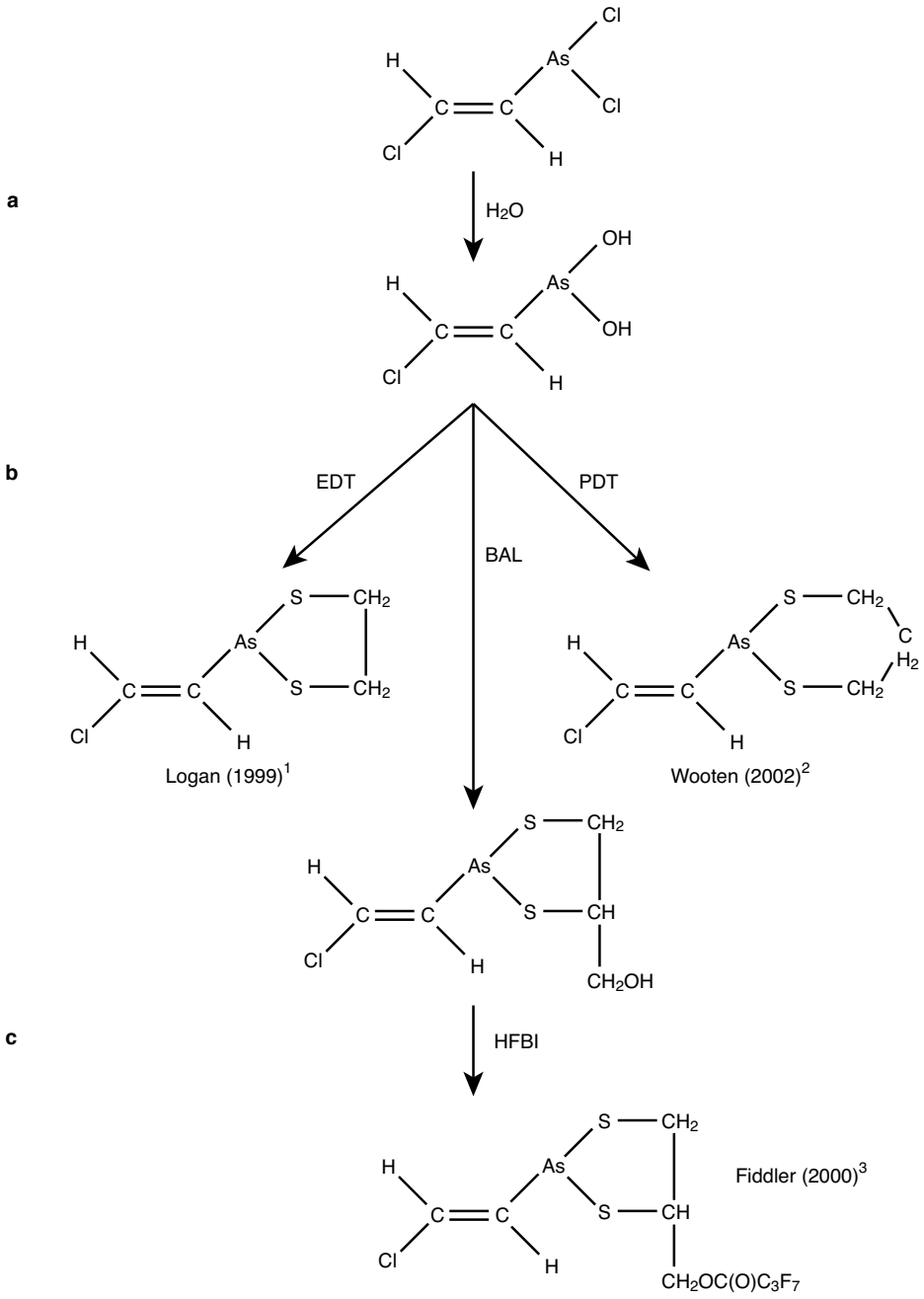


Fig. 22-12. Published analytical approaches for the analysis of chlorovinylarsonous acid in urine. **(a)** Reaction of lewisite (trans isomer shown) with water to form chlorovinylarsonous acid. **(b)** Reactions of chlorovinylarsonous acid with thiol compounds ethanedithiol, propanedithiol, and British anti-Lewisite.[1,2,3] **(c)** Derivatization using HFBI.[3]
BAL: British anti-Lewisite
EDT: ethanedithiol
H_2O: dihydrogen monoxide; water
HFBI: heptafluorobutyryl imidazole
PDT: propanedithiol
Data sources: (1) Logan TP, Smith JR, Jakubowski EM, Nielson RE. Verification of lewisite exposure by the analysis of 2-chlorovinyl arsonous acid in urine. *Toxicol Meth*. 1999;9:275–284. (2) Wooten JV, Ashley DL, Calafat AM. Quantitation of 2-chlorovinylarsonous acid in human urine by automated solid-phase microextraction-gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002;772:147–153. (3) Fidder A, Noort D, Hulst AG, de Jong LP, Benschop HP. Biomonitoring of exposure to lewisite based on adducts to haemoglobin. *Arch Toxicol*. 2000;74:207–214.

EXHIBIT 22-4

SAMPLE PREPARATION METHODS FOR GAS CHROMATOGRAPHIC/MASS SPECTROMETRIC ANALYSIS OF CHLOROVINYLARSONOUS ACID

SPE procedure of Logan et al for CVAA in urine:

- Precondition a C18 SPE cartridge with methanol followed by water.
- Adjust pH of 1 mL of urine to pH 6 using HCl.
- Add PAO as an internal standard.
- Transfer sample to C18 SPE cartridge for sample cleanup/extraction.
- Elute analytes using methanol.
- Evaporate to dryness under nitrogen.
- Reconstitute with ethanol containing EDT.
- Analyze using GC-MS with EI ionization.[1]

SPME headspace procedure of Wooten et al for CVAA in urine:

- Place 1 mL of urine into a 10-mL vial.
- Add ammonium acetate buffer-water solution.
- Add PAO as an internal standard.
- Add PDT in order to derivatize CVAA and PAO.
- Crimp-seal vial and heat at 70°C for 20 min.
- Insert a 100-μM poly(dimethylsiloxane) SPME fiber into the sample vial headspace for 10 min.
- Remove the SPME fiber from the vial and insert into the injection port of a GC-MS with EI ionization.[2]

SPE procedure of Fidder et al for free and bound CVAA in blood:

- Add 2,3-dimercapto-1-propanol (BAL) to a 2-mL blood sample. The BAL causes displacement of bound CVAA and derivatizes both the free and released CVAA.
- Add phenylarsine-BAL as an internal standard.
- Shake the sample at room temperature overnight.
- Dilute the sample with water.
- Transfer sample to C18 SPE cartridge for sample cleanup/extraction.
- Elute the analytes from the cartridge using dichloromethane/acetonitrile mixture.
- Concentrate the extracted sample to dryness.
- Redissolve in toluene.
- Add HFBI to derivatize the BAL hydroxyl group.
- Incubate the sample for 1 hour at 50°C and then cool to room temperature.
- Wash with water and then dry over $MgSO_4$.
- Analyze using GC-MS with EI ionization.[3]

BAL: British anti-Lewisite
CVAA: chlorovinylarsonous acid
EDT: ethanedithiol
EI: electron impact
GC: gas chromatography
HCl: hydrochloric acid
HFBI: heptafluorobutyryl imidazole
MS: mass spectrometry
PAO: phenylarsine oxide
PDT: 1,3-propanedithiol
SPE: solid phase extraction
SPME: solid phase microextraction

Data sources: (1) Logan TP, Smith JR, Jakubowski EM, Nielson RE. Verification of lewisite exposure by the analysis of 2-chlorovinyl arsonous acid in urine. *Toxicol Meth*. 1999;9:275–284. (2) Wooten JV, Ashley DL, Calafat AM. Quantitation of 2-chlorovinylarsonous acid in human urine by automated solid-phase microextraction-gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002;772:147–153. (3) Fidder A, Noort D, Hulst AG, de Jong LP, Benschop HP. Biomonitoring of exposure to lewisite based on adducts to haemoglobin. *Arch Toxicol*. 2000;74:207–214.

urine samples. The blood assay for both bound and free CVAA will potentially provide a longer opportunity for retrospective confirmation of exposure (based on one animal study), but also indicates a substantial decrease (90%) in concentration levels observed over a 10-day period.

CYANIDE

Background

Cyanide is an important industrial chemical that has many uses. It is produced in large quantities across the world and people are exposed to cyanide in a variety of ways. Many plants, including cassava roots, lima beans, and bamboo shoots, contain small amounts of cyanogenic glycosides.[131–133] Polymers that contain carbon and nitrogen release cyanide when burned. For example, large amounts of cyanide can be released during house and forest fires and from tobacco smoke (100–1600 parts per million [ppm]).[131] Some drugs also contain cyanide or have active ingredients that are converted to cyanide in the body, including sodium nitroprusside, which is given for the critical care of hypertension.[131] Synonyms for hydrogen cyanide (HCN) include prussic acid, formic anamminide, and fromonitrile. Deaths due to cyanide intoxication are usually due to the inhalation of HCN or the ingestion of cyanide salts. The signs of cyanide poisoning include headache, nausea, vomiting, tachypnoea, dyspnoea to convulsion, unconsciousness, and death.[131, 134–137]

Cyanide in the Human Body

Cyanide, the CN- ion, exists as HCN in the body at physiological pH. The mechanism of cyanide toxicity is believed to be the inactivation of iron III (ferric) enzymes in the body. The inhibition of cytochrome oxidase, which disrupts mitochondrial oxidative phosphorylation, is thought to be the most important mechanism for cyanide toxicity.[131, 138–141] Cyanide also binds to the hemoglobin in erythrocytes.[142] The binding of cyanide to iron III enzymes and proteins is reversible.[140,141]

At low concentrations, 93% to 99% of total cyanide is bound to methemaglobin (metHb) in the erythocytes.[131] The metHb is the deoxygenated iron III form of hemoglobin. Cyanide has a very high affinity to metHb and a low affinity to the oxygenated form of hemoglobin. Usually less than 1% of the total hemoglobin is in the metHb form, so at increased concentrations of cyanide in the blood, a larger amount is found in the serum. In tissue, cyanide binds to the heme group in mitochondrial cytochrome oxidase and inhibits electron transport.[140,141] All tissues are affected by this enzymatic inhibition, but especially those that require high amounts of oxygen and adenosine 5′-triphosphate. Other proteins and enzymes are also affected by cyanide, such as superoxide dismutase and xanthine oxidase, and may also contribute to cyanide's toxic effects.[131]

Cyanide's metabolism in the body is very rapid and can occur at 0.017 mg/min/kg.[131] The most common form of metabolism is the conversion to thiocyanate (SCN^-), which is then excreted via the kidneys.[143,144] The mitochondrial enzyme rhodanese (thiosulfate sulfur transferase) is thought to be the main catalyst for the formation of SCN^-, but β-mercaptopyruvate-cyanide transferase can transform cyanide to SCN^- through a different route.[141,145] The conversion to SCN^- is thought to be limited by the amount of thiosulfate. Cysteine, cystine, GSH, and β-mercaptopyruvic acid can also be sulfur sources.[131, 141,145] The reaction with cystine is also an important pathway leading to 15% of the total cyanide dose excreted in the urine as 2-iminothiozoline-4-carboxylic acid.[144] Other elimination pathways are the exhalation of HCN and oxidation to cyanate and reactions with vitamin B_{12} to form cyanocobalamin.[146]

Analytical Methods

Several matrices have been used to assess cyanide exposure. Whole blood is the most common, but measurements have been made in serum, plasma, saliva, tissues, gastric aspirate, and urine.

Whole Blood

Whole blood has been the matrix of choice, thus far, to determine cyanide exposure in humans. However, there are problems associated with cyanide and SCN^- measurements in blood. Sample collection, storage, and preparation are very important. Different levels of cyanide have been found in samples collected from different vessels (venous, arterial, and ventricular). Different whole blood samples can contain different amounts of metHb, which has a high affinity to cyanide. Also, free HCN may be more important because it is the form that reacts with cytochrome oxidase and causes the most significant adverse health effects.[131] Storage of the whole blood is also critical but not well understood. Some studies have shown an increase in cyanide of up to 40% upon storage of whole blood for 1 week and a 14% increase after 1 day at 4°C, while others have shown a decrease of 20% to 30% within 1

TABLE 22-13

ANALYTICAL METHODS FOR DETERMINING CYANIDE IN BIOLOGICAL SAMPLES[1]

Sample Matrix	Preparation Method	Analysis Method	LOD	Percent Recovery
Blood	Separation in MDC; derivatization	Spectrophotometry	0.1 ppm	NR[2]
Blood	Separation in MDC; derivatization	Spectrofluorometry (total CN)	0.025 ppm	NR[3]
Plasma	Deproteination with TCA; derivatization	Spectrophotometry (SCN-CN determination)	~ 0.07 ppm	96 (SCN)[4]
Erythrocyte suspension	Sample purged; absorption of HCN in NaOH; oxidation of SCN	Spectrophotometry (SCN-CN determination)	NR	93–97[5]
Blood cells	Centrifugation to separate cells; extraction; derivatization	HPLC-fluorescence detection	0.002 ppm	83[6]
Blood	Acidification	Headspace GC-NPD	~ 0.03 ppm	NR[7]
Blood	Acidification; derivatization	Headspace GC-ECD	0.1 ppm	NR[8]
Blood	Separation in MDC; color development	Spectrophotometry	~ 0.07 ppm	NR[9]
Blood	Incubation of acidified sample	GC-NPD	0.001 ppm	NR[10]
Blood	Separation in MDC; absorption in methemoglobin	Spectrophotometric (free CN)	0.4 ppm	~ 80[11]
Blood	Acidification	GC-NPD	0.014 ppm	86–99[12]
Blood	Microdiffusion, derivatization	Isotope dilution LC-MS	5 ppb	N/A[13]
Blood and liver	Sample digestion; treatment with lead acetate; absorption with NaOH	Specific ion electrode (total CN)	0.005 ppm	100–109[14]
Blood and urine	Separation in MDC; derivatization	Spectrofluorometric	0.008 ppm	66–83 (blood) 76–82 (urine)[15]
Urine	Dilution; derivatization	Spectrophotometry (SCN-CN determination)	~ 0.07 ppm	88 (SCN)[16]
Saliva	Derivatization	HPLC-UV (SCN)	2 ng (on instrument)	95–99[17]
Serum, urine, saliva	Extraction of buffered sample with isoamyl acetate	Flame AAS (SCN)	0.004 ppm	96–102[18]
Serum	Addition of acetonitrile; centrifugation; separation	Spectrophotometry (SCN)	0.3 ppm	94[19]
Urine, saliva	Basify; derivatization; extraction; back extraction	GC-ECD (SCN)	~ 0.033	83–106[20]
Urine, saliva	Dilution; filtration	Ion chromatography-UV (SCN)	0.02	95–101[21]
Urine	Ion chromatography; acidification; derivatization	Spectrophotometry (SCN)	~ 0.145 ppm (lowest reported)	NR[22]
Urine	Dilution; solid phase extraction	Suppressed ion chromatography with conductivity detection	~ 0.011 ppm	NR[23]
Urine (2-aminothiozoline-4-carboxylic acid)	Cation exchange; reduction; derivatization	Suppressed ion chromatography with fluorescence detection	~ 0.03 ppm	NR[24]
Urine (2-aminothiozoline-4-carboxylic acid)	Solid phase extraction and derivatization	Isotope dilution GC-MS	25 ppb	N/A[25]

(**Table 22-13** *continues*)

Table 22-13 *continued*

AAS: atomic absorption spectrometry
ATC: 2-amino-thiazoline-4-carboxylic acid
CN: cyanide
ECD: electron capture detection
GC: gas chromatography
GC-MSD: gas chromatography-mass selective detection
HCN: hydrogen cyanide
HPLC: high-performance liquid chromatography
LC: liquid chromatography
LOD: limit of detection
MDC: microdiffusion cell
NaOH: sodium hydroxide
NPD: nitrogen phosphorus detection
NR: not reported
ppb: parts per billion
ppm: parts per million
SCN: thiocyanate
TCA: trichloroacetic acid
UV: ultraviolet absorbance detection

Data sources: (1) US Department of Health and Human Services, Public Health Services, Agency for Toxic Substances and Disease Registry. Toxicological profile for cyanide. Atlanta, Ga: USDHHS; 1997. (2) Morgan RL, Way JL. Fluorometric determination of cyanide in biological fluids with pyrudixal. *J Anal Toxicol.* 1980;4:78–81. (3) Ganjeloo A, Isom GE, Morgan RL, Way JL. Fluorometric determination of cyanide in biological fluids with *p*-benzoquinone. 1980;55:103–107. (4) Pettigrew AR, Fell GS. Simplified colorimetric determination of thiocyanate in biological fluid, and its application to investigation of the toxic amblypias. *Clin Chem.* 1972;18:996–1000. (5) McMillan DE, Svoboda AC 4th. The role of erythrocytes in cyanide detoxification. *J Pharmacol Exp Ther.* 1982;221:37–42. (6) Sano A, Takimoto N, Takitani S. High-performance liquid chromatographic determination of cyanide in human red blood cells by pre-column fluorescence derivitization. *J Chromatogr.* 1992;582:131–135. (7) Levin BC, Rechani PR, Gruman JL, et al. Analysis of carboxyhemoglobin and cyanide in blood of victims of the DuPont Plaza Hotel fire in Puerto Rico. *J Forensic Sci.* 1990;35:151–168. (8) Odoul M, Fouillet B, Nouri B, Chambon R, Chambon P. Specific determination of cyanide in blood by headspace gas chromatography. *J Anal Toxicol.*1994;18:205–207. (9) Laforge M, Buneaux F, Houeto P, Bourgeios F, Bourdon R, Levillain P, et al. A rapid spectrophotometric blood cyanide determination applicable to emergency toxicology. *J Anal Toxicol.*1994;18:173–175. (10) Seto Y, Tsunoda N, Ohta H, et al.Determination of blood cyanide by headspace gas chromatography with nitrogen phosphorus detection and using a megabore capillary column. *Analytica Chimica Acta.*1993;276:247–259. (11) Tomoda A, Hashimoto K. The determination of cyanide in water and biological tissues by methemoglobin. *J Hazardous Materials.* 1991;28:241–249. (12) Calafat AM, Stanfill SB. Rapid quantitation of cyanide in whole blood by automated headspace gas chromatography. *J Chromatography B Analyt Technol Biomed Life Sci.* 2002;772:131–137. (13) Tracqui A, Raul JS, Geraut A, Berthelon L, Ludes B. Determination of blood cyanide by HPLC-MS. *J Anal Toxicol.* 2002;26:144–148. (14) Egekeze JO, Oehme FW. Direct potentiometric method for the determination of cyanide in biological materials. *J Anal Toxicol.* 1979;3:119–124. (15) Sano A, Takezawa M, Takitani S. Spectrofluorometric determination of cyanide in blood and urine with naphthalene-2,3-dialdehyde and taurine. *Anal Chim Acta.* 1989;225:351–358. (16) Pettigrew AR, Fell GS. Simplified colorimetric determination of thiocyanate in biological fluid, and its application to investigation of the toxic amblyopias. *Clin Chem.* 1972;18:996–1000. (17) Liu X, Yun Z. High-performance liquid chromatographic determination of thiocyanate anions by derivatization with pentafluorobenzyl bromide. *J Chromatogr A.* 1993;653:348–353. (18) Chattaraj S, Das AK. Indirect determination of thiocyanate in biological fluids using atomic absorption spectrometry. *Spectrochica.* 1992;47:675–680. (19) Li HZ, Bai G, Sun RM, Du LK. Determination of thiocyanate metabolite of sodium nitroprusside in serum by spectrophotometry. *Yao Xue Xue Bao.* 1993:28:854–858. (20) Chen SH, Wu SM, Kou HS, Wu HL. Electron-capture gas chromatographic determination of cyanide, iodide, nitrite, sulfide and thiocyanate anions by phase-transfer-catalyzed derivatization with pentafluorobenzyl bromide. *J Anal Toxicol.* 1994;18:81–85. (21) Michigami Y, Fujii K, Ueda K, Yamamoto Y. Determination of thiocyanate in human saliva and urine by ion chromatography. *Analyst.* 1992;117:1855–1858. (22) Tominaga MY, Midio AF. Modified method for the determination of thiocyanate in urine by ion-exchange chromatography and spectrophotometry. *Rev Farm Bioquim Univ Sao Paulo.* 1991;27:100–105. (23) Miura Y, Koh T. Determination of thiocyanate in human urine samples by suppressed ion chromatography. *Anal Sci 7.* 1991;(Suppl Proc Int Congr Anal Sci, 1991, Pt 1):167–170. (24) Lundquist P, Kagedal B, Nilsson L, Rosling H. Analysis of the cyanide metabolite 2-aminothiazoline-4-carboxylic acid in urine by high-performance liquid chromatography. *Anal Biochem.* 1995;228:27–34. (25) Logue BA, Kirschten NP, Petrovics I, Moser MA, Rockwood GA, Baskin SI. Determination of the cyanide metabolite 2-aminothiazole-4-carboxylic acid in urine and plasma by gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2005;819:237–244.

day at 4°C and continued decreases over 2 weeks. A decrease in cyanide levels was seen when whole blood was stored at 2°C. Results from storage at – 20°C has been disputed, with some studies showing up to 3.5 times the original cyanide levels and others showing no change.[147] These differences appear to be independent of the original cyanide concentrations.[148]

Prior to detection, cyanide must first be separated from hemoglobin. This separation is most often done with sulfuric acid and followed by microdiffusion in a Conway cell, although there have been some problems associated with the Conway cell. Other methods use acid to release the cyanide followed by a variety of techniques. Most of these methods are colorimetric and rely on the König reaction to produce a dye that is quantified using spectrophotometry. Cyanide and SCN^- both react and must be separated using microdiffusion or distillation. Additionally, most of these methods are time consuming and lack specificity or sensitivity. Detection limits using this approach are

generally in the high parts-per-billion range. There have also been assays developed that acidify the solution and then sample the head space for HCN followed by GC with a nitrogen-phosphorus detector or derivatization followed by GC with an electron capture detector. Detection limits by nitrogen-phosphorus detection and electron capture detector have been in the high parts-per-billion range. Cyanide has been measured in RBCs by high-performance LC after derivatization with fluorescence detection.

Urine, Saliva, and Serum or Plasma

Methods to detect cyanide exposure in human urine, saliva, and either serum or plasma have concentrated on SCN^-. These methods include derivatization with high-performance LC with ultraviolet detection, derivatization with spectrophotometric detection, or GC with electron capture detection. Some urine methods have measured the urinary metabolite 2-aminothiozoline-4-carboxylic acid.

Reference Range Values

Reference range values for cyanide in various matrices tend to vary greatly depending on the study and on the method of analysis. Thus, a reference range needs to be established for any method. In some of the studies, the range of blood cyanide levels in normal populations is less than 150 parts per billion and urinary SCN^- less than 1.0 mg/mL.[131] Smokers have much higher cyanide levels than nonsmokers; in some smokers blood cyanide levels as high as 500 ng/mL have been reported, which is 50 times higher than that typically reported for nonsmokers (Table 22-13).[131]

PHOSGENE

Background

Phosgene, also known as carbonyl chloride, was used extensively in World War I and caused more deaths than any other agent.[149] It is now a widely used industrial chemical. In 2002 the global production of phosgene was estimated to be over 5 million metric tons,[150] most of which is consumed at the production site.[151] The first synthesis of phosgene was performed in 1812 by exposing a mixture of chlorine and carbon monoxide (CO) to sunlight.[152] During World War I phosgene was produced in bulk by the reaction of chlorine and CO in the presence of activated carbon catalyst.[152] Phosgene is generally produced in the same way today but with higher efficiency because of newer high-surface–area catalysts.[153] Phosgene is an important intermediate in many industrial products, including insecticides, isocyanates, plastics, dyes, and resins.[150] Additionally, phosgene is formed during the combustion of chlorinated hydrocarbons during fires,[154] and by the photooxidation of chlorinated solvents in the atmosphere.[155]

At room temperature (20°C) phosgene is a fuming liquid with a vapor pressure of 1180 mm Hg and boiling point of 7.6°C. The gas is heavier than air, with a relative density ratio of 4.39 at 20°C. This density allows phosgene gas to collect in low-lying areas. Phosgene's odor is somewhat sweet and resembles that of fresh cut grass or hay. At higher concentrations the odor becomes pungent or burning and causes rapid olfactory fatigue.[156]

Exposure to phosgene gas causes irritation to the eyes, nose, throat, and respiratory tract. Higher phosgene gas exposures can lead to pulmonary edema and death. Exposure to liquid phosgene by direct skin or eye contact is rare but is thought to produce localized severe burns.[156] Inhalation is the most toxic exposure route for phosgene and the route thought to have caused most of the injuries and deaths during World War I. A patient who has inhaled phosgene requires advanced treatment techniques.

Phosgene is also a powerful acylating agent that reacts with nucleophiles such as amines, sulfides, or hydroxyls. Phosgene's toxicity was originally thought to be due to HCl generation during the reaction of phosgene with moisture in the body. Later, acylation reactions were found to be responsible for a majority of phosgene's toxic effects. Phosgene is greater than 800 times more toxic than HCl. Free amines protect against phosgene poisoning but not against the toxic effects of HCl, phosgene inhibits coenzyme I and HCl does not, and chemically similar compounds (such as ketene) that do not have chlorine to generate HCl have similar toxicity to phosgene.[157] Furthermore, phosgene's rate of reaction with free amines has been found to be much higher than its rate of reaction with water. In a solution of aniline in water, phosgene reacts almost exclusively with the aniline.[152]

Most of the data on health effects from phosgene are from inhalation exposures. The regulatory threshold limit value (8-hour, time-weighted average) for phosgene has been established by the National Institute for Occupational Safety and Health, an institute within the Centers for Disease Control and Prevention, as 0.1 ppm[158]; the 60-minute and 24-hour emergency exposure limits are 0.2 ppm and 0.02 ppm, respectively.[155]

The estimated LCt_{50} of phosgene concentration in air related to exposure time through inhalation is 500 ppm/min.[156] The aerosol exposure that is lethal to 100% of the exposed population (LCt_{100}) is estimated to range from 1,300 ppm/min to 1,600 ppm/min.[159] The nonlethal levels of phosgene are estimated to be less than 300 ppm/min, and 25 ppm/min is regarded as the threshold for lung damage.[156]

Phosgene odor can be recognized at levels greater than 1.5 ppm/min, with irritation in the mucus membranes occurring at 3 ppm/min or higher.[157] Exposure limits that cause adverse health effects can be reached either by longer exposure to lower concentration or shorter exposure to higher concentration. In one study, however, workers exposed to daily phosgene concentrations above 1 ppm but less than 50 ppm showed no difference in mortality or morbidity compared to workers in the same plant who were unexposed.[160]

The concentrations of phosgene in air that cause acute effects have been studied in many animal models. A review of previous animal studies in the literature was performed by Diller and Zante to estimate the approximate inhalation-dose–toxicity relationship for many species of animals.[161] In this report, animal LCt_{50} ranged from approximately 200 ppm/min for cats to 2000 ppm/min for goats. Guinea pigs and mice had the same approximate value as humans, 500 ppm/min. Dogs and rabbits had higher LCt_{50} values than humans (1000 ppm/min and 1500 ppm/min, respectively), while nonhuman primates and rats had estimated lower LCt_{50} values than humans (300 ppm/min and 400 ppm/min, respectively).[161]

Phosgene Metabolism and Markers for Phosgene Exposure

Phosgene is very reactive and is believed to be quickly transformed in vivo. Phosgene reacts with amino, hydroxyl, and thiol groups. In the blood, it can react with a variety of proteins, including albumin and hemoglobin. It also reacts with GSH and cysteine. Because chloroform is metabolized to phosgene in the body, it is expected that low levels of phosgene's protein adducts and metabolites can be found in the general background population because of low-level incidental exposure to chloroform. Studies are needed to accurately determine this reference range.

Glutathione and Glutathione Adducts

Because phosgene is a highly reactive acylating agent, it is expected to interact with the body's antioxidant defense system. GSH is a tripeptide thiol consisting of cysteine, glutamic acid, and glycine, which serves as both a scavenger of reactive compounds to protect cells and as a store for cysteine moieties.[162] GSH is found in general concentrations in healthy adults in the millimolar range.[162,163] GSH can be oxidized to glutathione disulfide (GSSG), a simple dimer of GSH joined by a disulfide linkage. The ratio of the GSH redox couple in vitro has been determined to be between 100:1 and 10:1 GSH to GSSG.[162,163] When depleted, GSH fails to protect cellular oxidation and leads to irreversible oxidative damage.[164]

Phosgene was found to react with GSH to form an acylated dimer, diglutathionyl dithiocarbonate (GSCOSG; Figure 22-13).[165] This marker for phosgene metabolism was first discovered by Pohl et al [165] in the metabolism of chloroform in the liver, where phosgene was believed to be generated by enzymatic action from chloroform. The bisglutathione adduct GSCOSG was detected in vivo in the bile of rats exposed to chloroform and in vitro in rat liver microsomes. Pohl et al also found a decrease in GSH levels in the rats.[165] GSCOSG was found directly by mixing phosgene with GSH in buffer solution.[166]

The in-vivo generation of GSSG and CO has also been reported in the blood of mice exposed to haloforms.[167] The observation of GSSG and CO may be linked to the further metabolism of GSCOSG after generation, but there are no studies that have identified this potential relationship.

GSH is also found in tissue, so similar reactions with GSH and phosgene are expected in the bronchoalveolar region. In the excised lung tissue from rabbits and mice inhalationally exposed to phosgene, there was

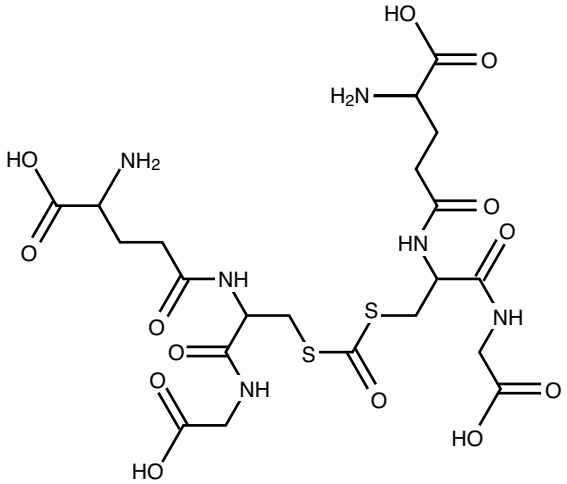


Fig. 22-13. Structure of diglutathionyl dithiocarbonate.

little change in tissue total GSH concentration, but the reduced GSH levels fell significantly and GSSG levels increased significantly.[168,169] In these studies, the reduced GSH as percent of total was 41% less than the control subject. The collection of lung tissue samples from humans that are thought to have been exposed to phosgene is impractical, but the collection of bronchoalveolar lavage fluid is possible. Sciuto has reported on the concentrations of GSSG, GSH, and total GSH in the bronchoalveolar lavage fluid of rodents with severe changes in the GSH redox state following phosgene inhalation.[170,171] Increases in total GSH levels were observed in both studies, but the redox state of the GSH was not reported. It can be anticipated that increased levels of the oxidized form of GSH would be a better indicator of phosgene exposure, but at this time only total GSH has been correlated with phosgene inhalation in lavage fluid. It should also be noted that in Sciuto's reported studies, there was no discrimination between GSSG and GSCOSG. Because these two species are similar in structure, it is unclear whether the reported detection of GSSG includes some component from GSCOSG.

The detection of GSCOSG, increased GSSG levels, and decreased GSH levels following phosgene exposure may hold promise for detecting phosgene exposure, but there are considerable obstacles to some of these approaches. The levels of total GSH and changes in GSH redox state in animal models were determined shortly after exposure to phosgene; however, the lifetime of GSCOSG is not known, and even though the levels of total GSH in mice exposed to phosgene were significantly higher than in controls up to 24 hours postexposure, the GSH levels were only marginally above controls at times up to 7 days postexposure.[171] Furthermore, there are large variations in GSH and GSSH levels in human subjects, so predetermined baseline levels should be required for each patient.[162,163] The formation of GSCOGS may be specific for phosgene (or chlorinated compounds that metabolize to phosgene). A reference range study of GSCOSG levels in people with no known exposure to phosgene is required to use this biomarker for phosgene exposure.

Cysteine Adducts

Phosgene reportedly forms an adduct with the thiol amino acid cysteine.[172] Cysteine is a building block of GSH and is the rate-limiting step in generating GSH for antioxidant tissue protection.[173] As in the case of GSH, cysteine can be reduced to the disulfide cysteine dimer cystine. Cysteine is generated in the body by the conversion of methionine through the cystathionine pathway. Average concentrations of cysteine in humans are typically lower than GSH and have been approximated at 250 μm in plasma and 400 μm in urine.[174]

The reaction of phosgene and cysteine in vitro was first reported by Kubic and Anders, and the product was identified as 2-oxothiazolidine-4-carboxylic acid (known as OTZ, OTC, or procysteine).[172] OTZ was formed by incubating hepatic microsomal fractions with chloroform, nicotinamide adenosine dinucleotide phosphate, and cysteine (Figure 22-14).[172] Isotopic studies showed that chloroform was first metabolized to phosgene by cytochrome P-450. The phosgene then reacted with cysteine to form OTZ. Synthetic routes to OTZ also have indicated the generation from cysteine and phosgene.[175]

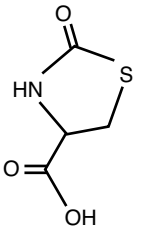
OTZ is a prodrug of cysteine and is rapidly converted by 5-oxoprolinase to cysteine in vivo.[173] Studies have indicated that the lifetime of OTZ in the human body varies from 3 to 8 hours.[173,176] OTZ's rapid elimination limits its usefulness as a biomarker for phosgene unless samples are obtained quickly after a suspected exposure.

Other possible markers for phosgene exposure may be the reduction of cysteine to its disulfide, cystine, upon exposure to phosgene. However, this reaction is not specific to phosgene exposure. The formation of the acylated dimer cysteine-CO-cysteine has been shown in vitro when cysteine is treated with phosgene in solution, and this reaction occurs even in the presence of GSH.[166]

Small Molecule Adducts

Inhaled phosgene reportedly reacts with other components in the lungs. In 1933 treatment of lung pulp with phosgene was found to form the chlorocarbonic ester of cholesterol.[177] Kling [177] also indicated that the hydrophilic character of the fats was destroyed in the cells because of the loss of free sterols and suggested that caused acute pulmonary edema.

Phosgene forms adducts with phospholipids. In a



Chemical Formula: $C_4H_5NO_3S$
Molecular Weight: 147.15

Fig. 22-14. Structure of 2-oxothiazolidine-4-carboxylic acid.

study of chloroform metabolism, a single phospholipid adduct was formed and it was thought to be related to phosphatidylethanolamine (PE) because PE was the only phospholipid found to be depleted.[178] In other studies on the metabolism of chloroform, a single phosgene adduct was found and identified as an adduct of PE with the phosgene carbonyl group bound to the amine of the head group of PE.[179,180] Further work on this PE adduct characterized it as a dimer of PE, the two subsistents linked at the amine of the head group by the carbonyl from phosgene.[181] Furthermore, this adduct has been implicated as the critical alteration leading to cell death by chloroform metabolism.[182] Nonspecific increases in the phospholipid content of lavage fluid have been observed in rats 6 hours after exposure to inhalational phosgene, but specific adducts were not monitored.[183]

Macromolecule Adducts

Lung tissue damage after phosgene exposure, which leads to permeability of the blood-air barrier and pulmonary edema, is likely due, in part, to the reactions of phosgene with various macromolecules, including proteins. The permeability of the blood-air barrier also raises the possibility of phosgene entering the blood stream and forming adducts with blood proteins. Some work has been done developing methods for identifying and quantifying phosgene adducts with abundant blood proteins.

The initial evidence that phosgene reacts and forms an adduct with hemoglobin was from a study of the metabolism of chloroform. In this study, Pereira et al allowed chloroform to be metabolized with liver microsomes.[184] One of chloroform's initial metabolites is believed to be phosgene. The study authors identified N-hydroxymethyl cysteine by GC-MS, resulting from the reaction of phosgene of cysteine moiety in hemoglobin.[184] Additionally, Fabrizi et al identified phosgene adducts with petapeptides of lysozyme and the N-terminal peptide from human histone H2B, even when these peptides were treated with phosgene in solution in the presence of GSH, a known phosgene scavenger.[168] There is evidence that phosgene reacts and forms blood protein adducts in vivo. Sciuto et al reported spectrophotometric differences in plasma from mice, rats, and guinea pigs exposed to high doses of phosgene.[185] This study also suggested that phosgene could directly attack RBCs after observing the shifted fragility curve for erythrocytes.[185]

Noort et al have developed methods to detect and quantify phosgene adducts with both hemoglobin and albumin.[186] Treatment of whole blood with radioisotopically labeled phosgene showed that phosgene reacted with both albumin and hemoglobin and that there was a higher level of adduct formation with the albumin than with the hemoglobin. The analysis of the albumin-^{14}C–labeled phosgene adduct indicated that the major site of adduct formation was an intramolecular lysine-lysine adduct with a CO bridge.[186] Analysis of the hemoglobin-phosgene adduct showed the presence of a hydantoin function between the N-terminal valine and the amino portion of leucine in a fragment containing amino acids 1 to 5.[186]

Noort et al[186] were able to detect the phosgene-albumin adduct in whole blood that was treated with 1 μM phosgene and the phosgene-hemoglobin adduct in whole blood treated with 1 mM phosgene. The phosgene-albumin adduct was detectable at an order of phosgene concentration magnitude 3 times lower than the phosgene-hemoglobin adduct. The higher detection limits of the globin adduct were reportedly in part due to an interference of natural hydantoin function in the same position as the phosgene adduct.[186] Sample preparation for the more sensitive albumin-based method included isolating albumin by affinity chromatography, carboxymethylation, dialysis, and tryptic digestion. LC-MS-MS analysis was done using either a high-resolution MS instrument or by multiple reaction monitoring on a triple quadrupole mass spectrometer. The multiple reaction monitoring experiment observed the fragmentation of the doubly charged ion at 861 daltons (da) to both the 747.5 da and 773.6 da transitions. Noort et al estimate that the method in vivo should be able to detect a 320 mg/min/m^3 exposure, assuming 10% absorption of the dose by the blood, resulting in an adduct concentration of 1.3 μm, just above the detection limit for the albumin adduct.[186]

Other Indicators of Phosgene Exposure

Inhalational exposure to phosgene causes severe inflammation and tissue damage. There are many indicators for the biochemical changes caused by phosgene exposure that measure the biochemical changes in the tissues that are damaged by phosgene. These types of markers are not as specific for phosgene exposure as small molecule or protein adduct markers, but they can suggest that an individual has been exposed to phosgene and indicate the severity of the exposure.

Some of the most important nonspecific indicators for phosgene-mediated tissue damage are associated with inflammation. Sciuto et al reported that interleukin (IL)-6 levels were highly elevated in the bronchoalveolar lavage fluid from rodents exposed

to phosgene compared to levels in controls.[185] Levels of IL-6 were 16-fold higher 4 hours after the phosgene exposure, peaked at 12 hours, and were still over 100-fold higher than in controls 72 hours after phosgene exposure. Macrophage inflammatory protein was also affected in the rodents exposed to phosgene, showing a 10-fold increase compared to the control rodents 8 and 10 hours after the exposure, but macrophage inflammatory protein levels returned to near-normal levels 24 hours after the phosgene exposure.[185] Sciuto et al reported that changes in IL-4, IL-10, tumor necrosis factor, and IL-1β levels were minimal after phosgene exposure as compared to changes in IL-6 levels.[185]

Sciuto et al also studied changes in fluid electrolyte levels in blood and bronchoalveolar lavage fluid from mice following exposure to phosgene.[187] Blood levels of sodium, chloride, and calcium were unchanged after exposure to phosgene compared to controls, but the levels of potassium increased drastically and approached physiologically dangerous levels. In the bronchoalveolar lavage fluid, chloride levels were unchanged, sodium dropped between 4 and 12 hours, and both potassium and calcium levels increased significantly after 4 hours and by a factor of 3 after 8 hours, compared to nonexposed controls. The simplicity of determining electrolyte levels offers promise for suggesting exposure to phosgene, but is not specific for phosgene exposure and requires supporting analyses.

Because phosgene reacts rapidly with GSH, it is expected that enzymes responsible for maintaining GSH levels may also be affected, and their levels can be monitored to suggest phosgene exposure. Sciuto et al found levels of GSH peroxidase and GSH reductase significantly increased in the bronchoalveolar lavage fluid of mice exposed to 160 to 220 ppm/min phosgene.[171] The levels of GSH peroxidase and GSH reductase were measured by ELISA and were elevated up to 72 hours after exposure.[171]

The amount of total protein in bronchoalveolar lavage fluid may also be a marker for the extent of lung damage after a phosgene exposure. Sciuto found that levels of total protein in the lavage fluid of mice was dramatically increased following phosgene exposure and remained significantly elevated up to 7 days postexposure. Other reports by Sciuto have indicated similar increases in protein levels in bronchoalveolar lavage fluid and have suggested they are due to increased permeability of the blood-air barrier.[170] In an earlier study, Currie et al found significant increases in the concentration of bronchoalveolar lavage fluid protein immediately after a relatively low exposure (60 ppm/min).[188] Total protein levels in bronchoalveolar lavage fluid are not specific for phosgene exposure, but may be helpful to determine the extent of lung damage caused by it. A simple spectrophotometric assay is suitable to determine protein levels because the amount of protein in lavage fluid is nonspecific and measures only total protein levels.

3-QUINUCLIDINYL BENZILATE

Background

Commonly termed "QNB," 3-quinuclidinyl benzilate (BZ) is an anticholinergic glycolate that has been designated "agent BZ" by the military. BZ is the only known incapacitating agent that has ever been weaponized for use by the US military. That occurred in the early 1960s when BZ was produced at the Pine Bluff Arsenal between 1962 and 1965. BZ was subsequently dropped from the chemical arsenal for several reasons, including concerns about variable and unpredictable effects.

BZ's action is very similar to other anticholinergics such as atropine and scopolamine, differing mainly in potency and duration of effect. The lethal dose of an infectious organism required to produce infection in 50% of the population (ID_{50}) for BZ is about 6.2 μg/kg (about 500 ng/person). BZ is about 25 times as potent as atropine, but only about 3-fold more active than scopolamine. However, BZ's duration of action is typically much longer, and the uptake by an oral route is about 80% that of intravenous or intramuscular routes. Under optimum conditions, BZ is also about 40% to 50% as effective by inhalation as by injection. Initial symptoms typically occur 30 minutes to 4 hours after exposure. Full recovery may require 3 to 4 days.

BZ has a molecular weight of 337 g/mol and a melting point of 168°C. It is solid at room temperature and has a negligible vapor pressure. BZ is relatively stable and moderately resistant to air oxidation and moderate temperatures, and it undergoes hydrolysis in aqueous solution to produce benzylic acid and 3-quinuclidinol.

3-Quinuclidinyl Benzilate in the Human Body

Information on BZ in the body is limited. It can take 3 to 4 days before symptoms of BZ intoxication resolve. Apparently, most BZ is excreted by the kidneys and urine is the preferred analysis matrix. Benzylic acid and 3-quinuclidinol are the probable main metabolites

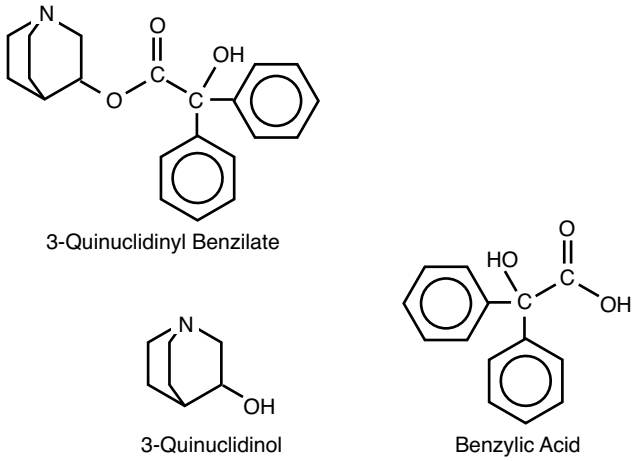


Fig. 22-15. Structure of 3-quinuclidinyl benzilate and the main metabolites 3-quinuclidinol and benzylic acid.

(Figure 22-15). It has been estimated that 3% of BZ in the body is excreted as the parent compound.

Analytical Methods

There are few references to the analysis of BZ in humans. BZ is frequently used by neuropharmacologists as a marker, but these methods are often not quantitative. In 1988 the United States began work on demilitarization of BZ stockpiles. As part of that work, the National Institute of Standards and Technology and the US Army Research and Development laboratory at Fort Detrick, Maryland, developed a method for monitoring workers. The method was based on GC-MS of the trimethylsilyl (TMS) derivatives and monitored all three analytes (BZ, benzylic acid, and 3-quinuclidinol). Detection limits were 0.5 ng/mL for BZ and 5 ng/mL for the hydrolysis products.[189]

SAMPLE CONSIDERATIONS

General

The following section describes the field collection, shipment, and storage of biomedical samples according to the guidance from the Centers for Disease Control and the United States Army Medical Research Institute of Chemical Defense. In most instances, blood (serum, plasma) and urine are the most commonly collected samples. Samples to be collected depend upon the specific method required by the assay. The information provided in this section is intended to provide general guidelines for sample collection, shipment, and storage and is not intended to be comprehensive. In all cases, questions regarding specific procedures to obtain and ship samples should be directed to the receiving laboratory by calling the laboratory or consulting its Web site before collecting the sample.

Urine

The collection of urine samples should be done under the close supervision of a healthcare provider or an unbiased observer to preclude the possibility of sample tampering. Care should be taken to ensure appropriate handling so as to minimize the chances for contamination from the environment or handling personnel. The urine should be collected immediately following the suspected exposure or at the earliest possible time. A midstream urine collection is desirable. If follow up is anticipated, additional samples should be obtained at 24 hours. Urine should be collected in clean urine cups or screw-capped plastic containers that can withstand freezing temperatures without splitting. A minimum of 25 to 30 mL should be collected. Urine samples should be frozen immediately (– 70°C or dry ice is preferred).

Whole Blood

It is recommended that samples of whole blood be handled cautiously from the start of the collection to maintain integrity and preclude the possibility of contamination, tampering, or mislabeling. All samples should be collected under the close supervision of a healthcare provider or physician and witnessed by an unbiased observer if possible. Samples should be obtained as soon as possible following the suspected exposure. Additional samples may be obtained for follow up. Blood should be collected in 5- or 7-mL blood tubes. Specific methods may require specific types of tubes, such as purple-top (ethylenediamine tetraacetic acid) tubes for plasma or the red-top, unopened, vacuum-fill tubes for serum.

For methods that analyze serum or plasma, it is useful to process whole blood samples by centrifugation followed by separation of the plasma or serum from the RBC pellet. The plasma or serum components can then be frozen (– 70°C or dry ice is preferred) and stored or shipped on dry ice. When on-site blood processing is not convenient, unprocessed whole blood samples should be stored immediately at 4°C. Packed RBCs may be stored at 4°C or frozen, depending on the properties of the analyte and the needs of the analytical method to be performed.

Preparing, Shipping, and Storing Specimens

Care must be taken not only when gathering samples for testing but when preparing them for transport. What follows are the basic guidelines for readying, transporting, and storing samples.

Labeling

Specimens should be labeled in accordance with the standing operating procedures of the receiving laboratory to ensure forensic integrity. The labels should be as comprehensive as possible and double-checked for accuracy. Label samples with the facility of origin and clear markings resistant to water and refrigeration or freezing conditions. Labels should also include patient identification information (name or other specific identifiers), date and time of collection, specimen identity, and some identification of the collector. A list of samples with the corresponding names of individuals should be maintained at the facility of origin and should also be included with the samples if they are shipped. Wrap each sample top with waterproof, tamper-evident, forensic evidence tape, being careful not to cover the sample identification labels.

Packaging

For blood, separate each tube from the others or wrap individually to prevent direct contact. Tubes should be placed in secondary packages, such as a divided box wrapped with absorbent material and sealed inside a plastic bag, other sealable containers, or individually wrapped tubes sealed inside a plastic bag. Place absorbent material between the primary receptacle and the secondary packaging. Use enough absorbent material to absorb the entire contents of primary receptacles. To facilitate processing and identification, package blood tubes so that similar tubes are packaged together (eg, all purple tops together). For urine, wrap frozen cups with absorbent material and place them into sealable secondary packaging, such as those described for blood. Do not ship frozen urine and blood in the same package.

Shipping Container

The shipping container should be a sealable, polystyrene foam or other insulated container capable of maintaining the contents at the preferred temperature for the specimens. For cushioning, place additional absorbent material in the bottom of the outer containers. For samples that require refrigeration conditions, such as whole blood, add a layer of frozen cold packs and place the secondary containers on top of the cold packs. Place additional cold packs or absorbent material between the secondary containers to reduce movement within the container. Lastly, place a layer of frozen cold packs on top of the secondary containers. When shipping frozen samples (plasma, serum, urine), add a layer of dry ice on top of the cushioning material in the bottom of the shipping container. Do not use large chunks of dry ice for shipment because they could shatter items during transport. Place additional absorbent material between wrapped urine cups to reduce their movement within the outer container. Finally, add an additional layer of dry ice.

Documentation (Shipping Manifest, Incident Report, Chain-of-Custody)

Prepare separate documentation for each container shipped. Prepare and place a shipping manifest designating sample identification numbers, quantity, and type in a zippered plastic bag on top of the specimens before closing and sealing the container. Maintain a copy of the manifest at the point of origin. Enclose an incident report form with as much information as possible describing the incident, providing the date and time of suspected exposure, detailing the onset and description of symptoms, sample collection time, and suspected agent involved. Include patient information, such as name, social security number, age, and gender, as well as a point of contact. The incident report form should be stored in a zippered plastic bag on top of the specimens before closing and sealing the container. A chain-of-custody form must also be included. Prepare a separate chain-of-custody form for samples in each container. Indicate sample identity, any pertinent descriptors, and the number of samples. Place the completed chain-of-custody forms in a plastic zippered bag on the outside of the shipping container.

Shipping

Close and secure the outer container with filamentous shipping or strapping tape. Affix labels and markings. Place a label on the outer container that indicates the proper name, "Diagnostic Specimens." For those containers with dry ice, place a Class 9 (dry ice) label on the outer container. This label must indicate the amount of dry ice in the container, the address of the shipper, and the address of the recipient. This label must be placed on the same side of the container as the "Diagnostic Specimens" label.

Storage

Upon arrival, the receiving laboratory should maintain a proper chain of custody. If the samples are not processed immediately, they should be stored as soon as possible after arriving at the receiving laboratory. Storing samples either before or after they are shipped should be in accordance with conditions dictated by the sample type. Blood should be stored refrigerated at 4°C. Plasma or serum should be stored frozen at –70°C. RBCs can be stored refrigerated at 4°C or frozen at –70°C; freezing is preferred for long-term storage. Avoid repeated cycles that move samples from frozen to thawed or refrigerated to room temperature.

SUMMARY

The general class of agents involved in severe intoxication (ie, OP nerve agents, vesicants, etc) can often be recognized by symptom presentation. However, testing is necessary to identify the specific agent involved. In cases where poisoning is suspected at low levels and symptoms do not clearly indicate intoxication with a specific chemical warfare agent, testing can provide additional information to help consider or rule out an exposure. In general, confirmatory analyses should not be initiated in the absence of other information that suggests a potential exposure has taken place; other evidence, such as patient signs or symptoms, environmental monitoring and testing, and threat intelligence information should also be considered. This information should be used to guide decisions about what agent or class of agents should be the focus of testing and it should ultimately be used in conjunction with test results to determine whether or not an exposure has occurred.

Analytical methods for verifying chemical agent exposure do not employ instrumentation that is routinely used for standard clinical testing, such as automated clinical analyzers. With the exception of cholinesterase analysis, instrumentation typically involves MS systems with either GC or LC techniques to separate the analyte from other matrix components. Although the methods are desirable because they afford a high level of confidence for identifying the analyte, they are time and labor intensive. Consequently the turnaround time for analyses is greater than that expected for standard clinical tests.

Chemical warfare agents have been used against both military and civilian populations. In many of these cases, healthcare providers have learned that it is critical to rapidly identify exposed personnel to facilitate appropriate medical treatment and support. Incidents involving large numbers of personnel have shown that is also important to determine those not exposed to avoid unnecessary psychological stress and overburdening the medical system. In addition to medical issues, the political and legal ramifications of chemical agent use by rogue nations or terrorist organizations can be devastating. Therefore, it is important that accurate and sensitive analytical techniques be employed and appropriately interpreted.

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