Verification of chemical warfare agent exposure in human samples

Paul W. Elsinghorst, Horst Thiermann, Marianne Koller

Institut für Pharmakologie und Toxikologie der Bundeswehr, München

Abstract

Aim: This brief presentation provides an overview of methods that have been developed for the verification of human exposure to chemical warfare agents.

Methods: GC–MS detection of nerve agents (V- and G-type) has been carried out with respect to unreacted agents as well as enzyme-bound species and metabolites. Methods involving direct SPE from plasma, fluoride-induced release of protein-bound nerve agents in plasma and analysis of their metabolites in plasma and urine have been developed. Exposure to blistering agents, *i.e.*, sulfur mustard, has been verified by GC–MS detection of the unreacted agent in plasma and by LC– and GC–MS analysis of its metabolites in urine.

Results: After incorporation nerve agents quickly bind to proteins, *e.g.*, acetylcholinesterase, butyrylcholinesterase or serum albumin, and only small parts remain freely circulating for a few hours (G-type) or up to 2 days (V-type). Concurrently they are converted to *O*-alkyl methylphosphonic acids by phosphotriesterases and/or simply by aqueous hydrolysis. As a result, different biomarkers can be detected depending on the time passed between exposure and sampling. Unreacted V-type agents can be detected in plasma for 2 days, the *O*-alkyl methylphosphonic acids in plasma for about 2–4 days and in urine for up to 1 week. Fluoride-induced release of protein-bound nerve agents can be carried out until 3 weeks post exposure. Unmetabolized sulfur mustard may be detected between 8 hours to 8 weeks in plasma, while no time frame has been reported for its metabolites in urine.

Conclusion: A set of validated techniques detecting exposure to chemical warfare agents has been established. Further methods are needed to provide a clear picture of their biomarkers.

1. Introduction

Although means of chemical warfare have been considered by political and military leaders since ancient times, the most dreadful substances in this respect originate from the 20th century (Fig. 1). During World War I the development of a large-scale production process for sulfur mustard, bis(2-chloroethyl)sulfide (HD, **1**), enabled first the German and later the British troops to deploy this compound as a blistering agent. Following in the advent of World War II, the G-type nerve agents tabun (1936, GA, **2**) and sarin (1938, GB, **3**) were discovered as part of a pesticide development project. Further compounds originating from pesticide research were soman (1944, GD, **4**), cyclosarin (1949, GF, **5**) and, later, the V-type compound VX (1952, **6**). During cold war two other substances were developed imitating VX, namely Russian VX (1963, VR, **7**) and Chinese VX (CVX, **8**). Although the distinction between G- and V-type nerve agents was initially based on a historic perspective (G: German, V: victory, venomous, viscious), G-type agents may be grouped as methylphosphonates (with the exception of tabun) and V-type agents as methylphosphonothioates.

While blistering agents exert their toxic potential by rather unspecific alkylation of DNA and proteins leading to downstream cellular damage, nerve agent toxicity originates from the very distinct interaction of the organophosphorus compounds with their target enzyme acetylcholinesterase. Phosphylation of the active site serine residue renders the enzyme inactive

with a dramatic impairment of cholinergic neurotransmission. Accordingly, symptoms relate to all cholinergic structures of the body, *e.g.*, neuromuscular transmission (seizures, paralysis, miosis) and secretion (lacrimation, salivation).

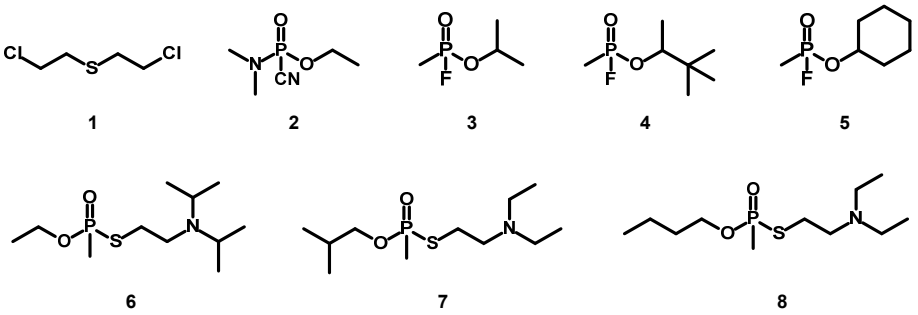


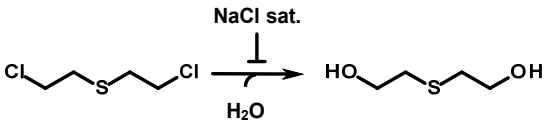
Fig. 1. Molecular structures of major chemical warfare agents.

In 1993 the Chemical Weapons Convention entered into force, which by today has been signed by 188 states parties. This convention bans all kinds of production, development, proliferation or use of chemical warfare agents, and schedules destruction of chemical warfare stock piles in the signatory states. To provide litigable proof of a potential human exposure towards either blistering and/or nerve agents suitable methods have been designed by several laboratories of the signatory states. These methods provide reliable results for both free and metabolized agents in different sample types [1].

2. Blistering Agents

2.1. Free Sulfur Mustard, Thiodiglycol, Thiodiglycol Sulfoxide

Following exposure to sulfur mustard the agent either quickly reacts with cellular nucleophiles like DNA or proteins or hydrolyzes to thiodiglycol, which subsequently oxidizes to give the corresponding sulfoxide. However, small amounts of sulfur mustard remain freely circulating and hydrolysis can be suppressed by addition of saturated NaCl (Fig. 2). The free agent may then be recovered from plasma by solid-phase extraction and subjected to GC–MS detection. Whereas thiodiglycol may also be analyzed by GC–MS following suitable derivatization, *e.g.*, silylation or pentafluorobenzoylation, the sulfoxide is preferably investigated by LC–MS as its extractability is very poor because of the highly polarized sulfoxide moiety [2].



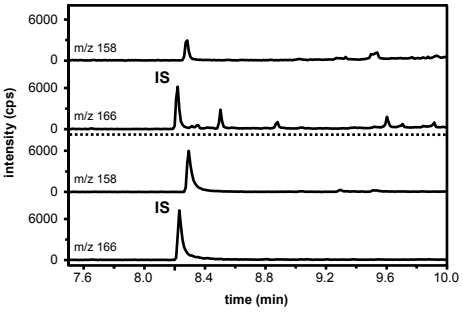
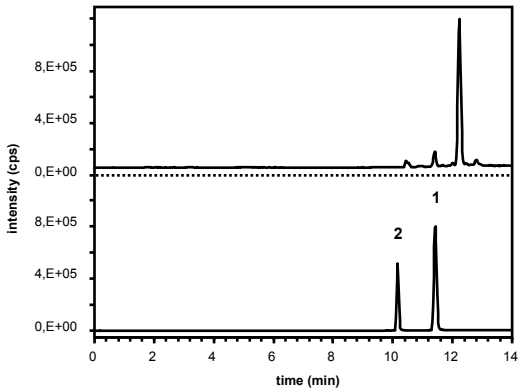


Fig. 2. GC–MS of native sulfur mustard; top: spiked human plasma, bottom: reference compounds (EI, IS: d_8-sulfur mustard, column: Optima 5 Accent; carrier: helium; injector: CIS; oven: 60 °C (2.1 min), 120 °C (20 °C/min), 125 °C (3 °C/min), 280 °C (30 °C/min).

While levels of free sulfur mustard are usually detectable for several days, its metabolites can be found in urine for up to 2 weeks. However, although sulfur mustard is rapidly metabolized after absorption it may reemerge from lipophilic tissues thus constantly replenishing free sulfur mustard levels.

2.2. β-Lyase Products

Besides hydrolysis to thiodiglycol sulfur mustard is extensively conjugated to glutathione. Subsequent cleavage of these glutathione adducts by β-lyase and oxidation of all primary metabolites provides two further metabolites, MSMTESE (1) and SBMSE (Fig. 3). These rather polar metabolites are amenable to LC–MS/MS analysis following simple solid-phase extraction from urine samples. Chromatographic separation allows separate quantification of either metabolite. To access these analytes by GC–MS the polar sulfoxide groups must be reduced. Selective reduction of the sulfoxides is achieved by reacting with titanium(III) chloride under acidic conditions. While this provides one common extractable analyte, 1,1'-sulfonylbis((2-methylthio)ethane), it does not allow to differentiate between MSMTESE and SBMSE [3].



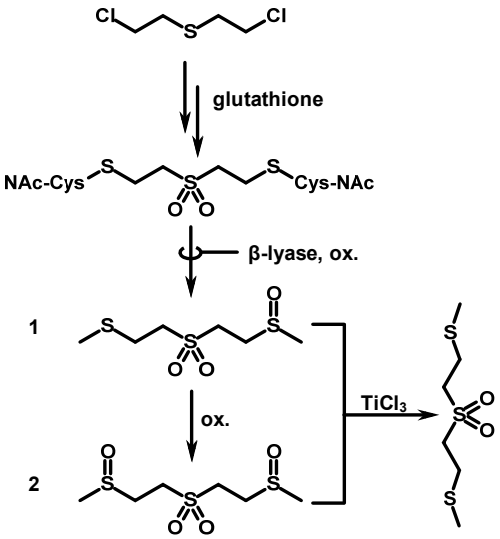


Fig. 3. LC–MS/MS detection of MSMTESE (**1**) and SBMSE (**2**); top: spiked human urine, bottom: reference compounds (ESI pos., column: Hypercarb 2.1 × 100 mm; solvent A: water, solvent B: methanol; gradient: 0-11 min 95% A, 12-20 min 5% A; post-column infusion: 400 mM NH_4COOH, flow: 200 µl/min, temperature: 35 °C).

3. Nerve Agents

3.1. Free G/V-Type Nerve Agents

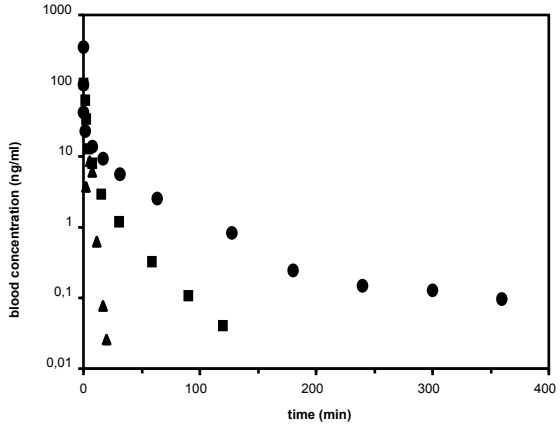


Fig. 4. Concentration-time course of VX (●), soman (■), and cyclosarin (▲) in blood of anesthetized, atropinized, and artificially ventilated guinea pigs after *i.v.* administration (reproduced from [4] and [5]).

After incorporation, nerve agents quickly bind to proteins, especially to the active site serine side chain of acetylcholinesterase and butyrylcholinesterase. But also other proteins like serum albumin are affected where non-catalytic tyrosine and serine residues are phosphylated. As a result only small parts of the incorporated nerve agents remain freely circulating. Samples taken within a few hours (G-type) or up to 2 days (V-type) may be analyzed by GC–MS after workup with protein precipitation ($HClO_4$) and subsequent solid-phase extraction (C_8).

3.2. Nerve Agent Metabolites

Both, aqueous hydrolysis and degradation by phosphotriesterases transforms G- and V-type nerve agents to *O*-alkyl methylphosphonic acids (with the exception of tabun). The substitution pattern of these metabolites allows the identification of the original nerve agent incorporated. *O*-Alkyl methylphosphonic acids may be recovered from either plasma or urine within one week post exposure (Fig. 5). Typical sample preparation involves protein precipitation by $HClO_4$ (plasma) or acidification by HCl (urine) followed by solid-phase extraction (ENV^+) and subsequent LC–MS/MS analysis.

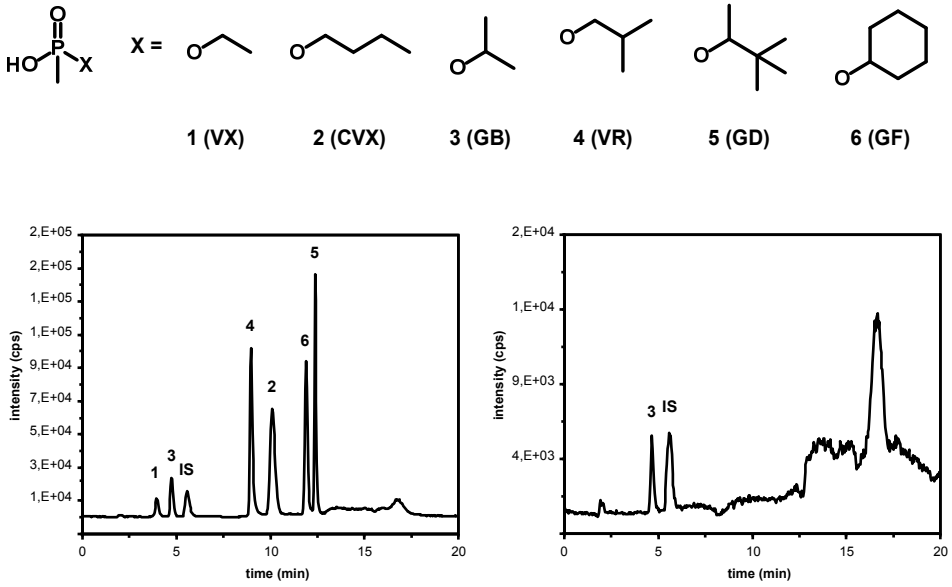


Fig. 5. top: Nerve agent metabolites (parent indicated); bottom/left: LC–MS/MS analysis of *O*-alkyl methylphosphonic acid metabolites (ESI neg., column: Hypercarb 2.1 × 100 mm; solvent A: 0.5% formic acid aq.; solvent B: acetonitrile; gradient: 0-3 min 100% A, 4-7 min 80% A, 9-16 min 20% A, 17-22 min 100% A; flow: 175 µl/min, temperature: 30 °C); bottom/right: detection of *O*-isopropyl methylphosphonic acid, the sarin metabolite, in spiked human urine (IS: *n*-butylphosphonic acid).

3.3. Fluoride-induced reactivation

As pointed out above, nerve agents rapidly bind to endogenous proteins as a result of the reaction of suitable amino acid residues (Ser, Tyr) attacking the activated phosphyl moiety releasing either fluoride (G-type) or thiolates (V-type). This reaction, however, can be reversed by excess fluoride rereleasing the phosphyl moiety in form of the corresponding fluorinated analogs (Fig. 6). These analogs may be recovered by SPE (ENV^+) for GC–MS detection up to several weeks post exposure [6].

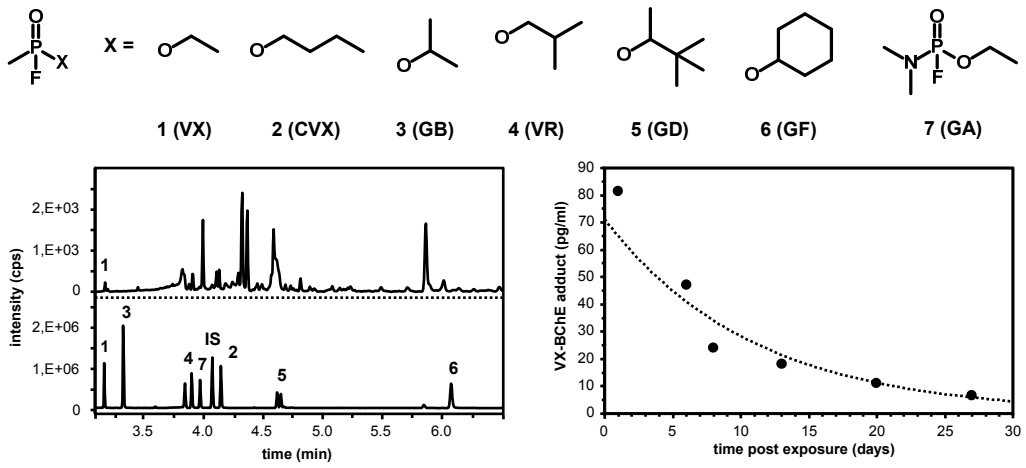


Fig. 6. top: Fluorinated nerve agent analogs (parent indicated); bottom/left: GC–MS detection of G/V-type nerve agents after fluoride induced release from plasma protein adducts; top: VX-spiked human plasma, bottom: reference compounds (EI, column: VF-5ms; carrier: helium; injector: CIS; oven: 60 °C (1.8 min), 120 °C (20 °C/min), 125 °C (3 °C/min), 200 °C (30 °C/min) (IS: DFP); bottom/right: the decay curve of human VX-BChE adducts obtained following an accidental VX exposure (reproduced from [7]).

4. References

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