

43. METHODS FOR RETROSPECTIVE DETECTION OF EXPOSURE TO TOXIC SCHEDULED CHEMICALS: AN OVERVIEW

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INTRODUCTION

Methods to analyze toxic scheduled chemicals and their decomposition products in environmental samples have been developed and are used, *e.g.*, to test the proficiency of laboratories to act as so-called designated laboratories for the OPCW. However, methods for such analyses in biological samples have only recently been developed, while the use or alleged use of chemical warfare (CW) agents in war and terrorism has clearly established an urgent need for biological markers of poisoning, *e.g.*, in blood and urine samples.

Retrospective detection of exposure to toxic scheduled chemicals can be useful for various reasons. First of all, such analytical methods can be used to establish firmly whether casualties have indeed been exposed to these chemicals, whereas dosimetry of the exposure will be a starting point for medical treatment of the intoxication. Second, these methods will be useful for verification of alleged non-adherence to the Chemical Weapons Convention. Especially in this application it appears that maximal retrospectively, preferably over a period of several months, is essential. Moreover, these methods can be used in a variety of other applications, *e.g.*, for health surveillance of workers in destruction facilities of CW agents and in forensic analyses in case of suspected terrorist activities.

In this report an overview is presented of the methods currently available for detection of exposure to sulfur mustard, nerve agents, lewisite and phosgene.

SULFUR MUSTARD

Sulfur mustard is a strong alkylating agent that reacts readily with nucleophiles under physiological conditions. The reaction products of sulfur mustard with these nucleophiles are all potential biological markers of human poisoning. Metabolites derived from an initial reaction with water and glutathione are excreted in urine (1-4). Adducts to DNA which may be present in various tissues and blood can conveniently be detected by using an immunochemical assay (5). In this section we will focus on adducts to hemoglobin and albumin, since it is expected that they are persistent and will allow retrospective detection.

Upon incubation of human blood with sulfur mustard, it appears that 20-25% of the dose was covalently bound to hemoglobin (6). The most abundant adduct was the histidine adduct (7). In addition, the adducts to cysteine, glutamic and aspartic acid and to the N-terminal valine residues were detected (7,8). As a biological marker of poisoning, N-alkylated N-terminal valine has the advantage that it can be selectively cleaved from hemoglobin by a modified Edman procedure using pentafluorophenyl isothiocyanate as reagent (9). Analysis of the resultant pentafluorophenyl thiohydantoin, using negative ion GC-MS-MS after further derivatisation with heptafluorobutyric anhydride, provided a very sensitive method for the detection of the N-alkylated valine (10). *In vitro* exposure of human blood to $\geq 0.1 \mu\text{M}$ sulfur mustard and *in vivo* exposure of guinea pigs could be detected employing this method. Moreover, the adduct could be detected in samples from victims of accidental exposure to sulfur mustard and CW casualties (11,12). Recently, a standard operating procedure (SOP) for determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin was developed. By using this SOP, it was found that the N-terminal valine adduct in globin of hairless guinea pigs and marmosets subsequent to I.V. administration of sulfur mustard (0.5 LD₅₀) is persistent for at least 56 and 94 days, respectively. The SOP could be properly set up and carried out at another institute (U.S. Army Medical Research Institute of Chemical Defense, Edgewood, MD, USA) within one working day.

Recently, sulfur mustard has been shown to alkylate a cysteine residue in human serum albumin (13). The site of alkylation was identified in a tryptic digest of albumin from blood exposed to [¹⁴C]sulfur mustard. A sensitive method for its analysis was developed based on Pronase digestion of alkylated albumin to the tripeptide S-[2-[(hydroxyethyl)thio]ethyl]-Cys-Pro-Phe, and detection using micro-LC-MS-MS. *In vitro* exposure of human blood to $\geq 10 \text{ nM}$ sulfur mustard could be detected employing this method. The analytical procedure was successfully applied to albumin samples from Iranian casualties of the Iraq-Iran war.

NERVE AGENTS

Five approaches have been explored in order to detect exposure to a nerve agent:

- (i) measurement of acetylcholinesterase (AChE) inhibition (14,15),
- (ii) detection of the intact agent,
- (iii) detection of hydrolysis product of nerve agents,
- (iv) analysis of phosphorylated butyrylcholinesterase (BuChE) and, more recently,
- (v) determination of phosphorylated serum albumin (16).

In this paper we will focus on items (iii) and (iv).

Hydrolysis products of nerve agents

Metabolism of phosphonofluoridates and V agents in mammals leads predominantly to hydrolysis products, *i.e.*, the corresponding O-alkyl methylphosphonic acid and a minor amount of methylphosphonic acid. Methods for analysis of these compounds are based on GC-MS (*e.g.*, 17-19) or on LC-MS (*e.g.*, 20-22). For instance, the hydrolysis product O-ethyl methylphosphonic acid has been determined by GC-MS, after derivatization, in serum collected from a victim poisoned by VX (23). Recently, a convenient and rapid LC tandem MS method was developed for quantitative determination of O-isopropyl methylphosphonic acid (IMPA), *i.e.*, the hydrolysis product of sarin, in blood and urine (24). Sample work-up was limited to a single extraction step. This method could be successfully applied to the analysis of serum samples from the victims of the Tokyo subway attack by the Aum Shinrykio sect and from an earlier incident at Matsumoto. It is envisaged that this method can be used for analysis of other hydrolyzed nerve agents as well.

Advantageously, the determination of hydrolysis products identifies the nerve agent except for its leaving group. However, the rather rapid elimination rate of the hydrolysis product from the organism (within several days) limits its use for retrospective detection of exposure.

Analysis of phosphorylated butyrylcholinesterase (BuChE)

In principle, organophosphate-inhibited BuChE in human plasma is a persistent and abundant source for biomonitoring of exposure to organophosphate anticholinesterases. Polhuijs et al. (25) developed a procedure for analysis of phosphorylated BuChE in plasma or serum samples, which is based on reactivation of the phosphorylated enzyme with fluoride ions: this converts the organophosphate moiety completely into the corresponding phosphofluoridate, which is subsequently isolated and quantitated. As for analysis of hydrolysis products this approach identifies the organophosphate except for its leaving group. Moreover, the extent of the organophosphate poisoning can be determined in this way. Furthermore, based on the minimal concentrations of phosphofluoridate that can be analyzed in blood, it can be calculated that inhibition levels $\geq 0.01\%$ of inactivated BuChE (*i.e.*, trace level exposure) should be quantifiable. Evidently, by analyzing the inhibited enzyme instead of the uninhibited enzyme, inhibitor levels that are several orders of magnitude lower can be quantified. The method is limited by spontaneous reactivation and aging (*i.e.*, loss of the alkyl moiety from the alkoxy moiety of the phosphyl group) of the phosphorylated enzyme and by the natural life span of the enzyme. Application of this method to serum samples of the victims from the Tokyo subway attack and of the Matsumoto incident yielded sarin concentrations in the range of 0.2-4.1 ng/mL serum. Evidently, these victims had been exposed to an organophosphate with the structure $iPrO(CH_3)P(O)X$, presumably with $X = F$ (sarin). A more laborious and qualitative method, reported by Nagao et al. (26) and by Matsuda et al. (27), is based on isolation and trypsinization of inhibited cholinesterases, subsequent treatment with alkaline phosphatase, followed by isolation, derivatization and GC-MS analysis of the released phosphyl moiety.

LEWISITE

Substantial stockpiles of the organoarsenical vesicant lewisite are present in the U.S.A. and in Russia. This may constitute a potential hazard for public health. The most generally applied method for determination of an arsenical is by atomic absorption spectrometry (AAS) after reduction of the compound to AsH_3 . However, this will only provide an indication for the presence of the element As.

Lewisite will rapidly hydrolyze to 2-chlorovinylarsonous acid (CVAA) in aqueous environment such as blood plasma. Consequently, analytical methods mainly focus on the determination of CVAA. For instance, it was shown (28-30) that CVAA could be isolated from serum and urine after addition of 1,2-ethanedithiol, followed by extraction of the resulting complex, which could be analyzed by GC-MS. These methods, however, do not allow retrospective verification of exposure, since CVAA is rapidly excreted into the urine.

In view of the high affinity of arsenic for thiol functions, it can be expected that lewisite, as well as CVAA, binds to cysteine residues of proteins. It was found that 25-50% of the dose was associated with globin, after treatment of human blood with 20 nM to 0.2 mM of [^{14}C]lewisite (31). The CVAA residues could be isolated from globin after

addition of 2,3-dimercaptopropanol (BAL), followed by extraction of the resulting lewisite-BAL complex, which could be sensitively analyzed by GC-MS after additional derivatization. The lowest detectable concentration of lewisite for in vitro exposure of human blood was determined to be 1 nM. A preliminary in vivo exposure was performed with guinea pigs. The amount of L1-BAL isolated from blood samples clearly decreased with increasing time after exposure, as should be expected. In the blood sample taken 10 days after exposure the amount of isolated BAL adduct had decreased to 10% of the amount at one day after exposure. The compound could only be detected in urine during the first 12 h after exposure, indicating the rapid excretion of unbound CVAA.

PHOSGENE

The pulmonary agent phosgene was used as a chemical weapon for the first time in WW I. Nowadays, it is an important intermediate for industrial production of insecticides, isocyanates, plastics, aniline dyes and resins, with an estimated yearly production of almost 1 billion pounds. Reliable diagnosis of exposure to phosgene other than observation of the developing edema by means of chest roentgenology is not available. It was recently found that phosgene binds effectively to albumin and hemoglobin upon in vitro exposure of human blood to [¹⁴C]phosgene (32). *Inter alia*, phosgene appears to crosslink the lysine residues 195 and 199 in human serum albumin. A mass spectrometric method was developed for analysis of the tryptic digest containing this intramolecular lysine-lysine adduct, which enabled the detection of exposure of human blood to $\geq 1 \mu\text{M}$ phosgene in vitro. Whether this method can be used for assessment of in vivo exposure will be determined in due course.

CONCLUSIONS

- Adducts with macromolecules such as proteins offer long lived biological markers of exposure, possibly up to several months.
- Gas or liquid chromatography combined with tandem mass spectrometry is the method of choice for unequivocal identification at trace levels.
- The discussed analytical methods, with the exception of ChE inhibition measurements and immunoassays, cannot easily be performed, require expensive equipment and strongly deviate from methods for analysis of environmental samples.

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REFERENCES

1. Black, R.M., and Read, R.W. (1988) *J. Chromatogr.* **449**, 261-270.
2. Black, R.M., Brewster, K., Clarke, R.J., Hambrook, J.L., Harrison, J.M., and Howells, D.J (1992) *Xenobiotica* **22**, 405-418.
3. Black, R.M., and Read, R.W. (1995) *J. Chromatogr. B* **665**, 97-105.
4. Black, R.M., and Read, R.W. (1995) *Xenobiotica* **25**, 167-173.
5. Van der Schans, G.P., Scheffer, A.G., Mars-Groenendijk, R.H., Fidder, A., Benschop, H.P., and Baan, R.A. (1994) *Chem. Res. Toxicol.* **7**, 408-413.
6. Noort, D., Verheij, E.R., Hulst, A.G., De Jong, L.P.A., and Benschop, H.P. (1996) *Chem. Res. Toxicol.* **9**, 781-787.
7. Noort, D., Hulst, A.G., Trap, H.C., De Jong, L.P.A., and Benschop, H.P. (1997) *Arch. Toxicol.* **71**, 171-178.
8. Black, R.M., Harrison, J.M., and Read, R.W. (1997) *Xenobiotica* **27**, 11-32.
9. Törnqvist, M., Mowrer, J., Jensen, S., and Ehrenberg L. (1986) *Anal. Biochem.* **154**, 255-266.
10. Fidder, A., Noort, D., De Jong, A. L., Trap, H.C., De Jong, L.P.A., and Benschop, H.P. (1996) *Chem. Res. Toxicol.* **9**, 788-792.
11. Black, R.M., Clarke, R.J., Harrison, J.M., and Read, R.W. (1997) *Xenobiotica* **27**, 499-512.
12. Benschop, H.P., Van der Schans, G.P., Noort, D., Fidder, A., Mars-Groenendijk, R.H., and De Jong, L.P.A. (1997) *J. Anal. Toxicol.* **21**, 249-251.
13. Noort, D., Hulst, A.G., De Jong, L.P.A., and Benschop, H.P. (1999) *Chem. Res. Toxicol.* **12**, 715-721.
14. Ellman, G.L., Courtney, K.D., and Anders, V. (1961) *Biochem. Pharmacol.* **7**, 88-95.
15. Worek, F, Mast, U. Kiderlen, D., Diepold, C., and Eyer, P. (1999) *Clin Chim Acta.* **288**, 73-90.

16. Black, R.M., Harrison, J.M., and Read, R.W. (1999) Arch. Toxicol. 73, 123-126.
17. Shih, M.L., Smith, J.R., McMonagle, J.D., Dolzine, T.W., and Gresham, V.C. (1991) Biol. Mass Spectrom. 20, 717-723.
18. Black, R.M., Clarke, R.J., Read, R.W., and Reid, M.T.J. (1994) J. Chromatogr. A 662, 301-321.
19. Fredriksson, S.-Å., Hammarström, L.-G., Henriksson, L., and Lakso, H.-Å. (1995) J. Mass Spectrom. 30, 1133-1143.
20. Tørnes, J.A. (1996) Rapid. Commun. Mass Spectrom. 10, 878-882.
21. Black, R.M., and Read, R.W. (1997) J. Chromatogr. A 759, 79-92.
22. Black, R.M., and Read, R.W. (1998) J. Chromatogr. A 794, 233-244.
23. Tsuchihashi, H., Katagi, M., Nishikawa, M., and Tatsuno, M. (1998) J. Anal. Toxicol. 22, 383-388.
24. Noort, D., Hulst, A.G., Platenburg, D.H.J.M., Polhuijs, M., and Benschop H.P. (1998) Arch. Toxicol. 72, 671-675.
25. Polhuijs, M., Langenberg, J.P., and Benschop, H.P. (1997) Toxicol. Appl. Pharmacol. 146, 156-161.
26. Nagao, M., Takatori, T., Matsuda, Y., Nakajima, M., Iwase, H., and Iwadate, K. (1997) Toxicol. Appl. Pharmacol. 144, 198-203.
27. Matsuda, Y., Nagao M., Takatori, T., Nijima, H., Nakajima, M., Iwase, H., Kobayashi, M., and Iwadate, K. (1998) Toxicol. Appl. Pharmacol. 150, 310-320.
28. Fowler, W.K., Stewart, D.C., Weinberg, D.S., and Sarver, E.W. (1991) J. Chromatogr. 558, 235-246.
29. Jakubowski, E.M., Smith, J.R., Logan, T.P., Wiltshire, N., Woodard, C.L., Evans, R.A., and Dolzine, T.W. (1993) Proceedings 1993 Medical Defense Bioscience Review Vol. I, 361-368.
30. Logan, T.P., Smith, J.R., Jakubowski, E.M., and Nielson, R.E. (1996) Proceedings 1996 Medical Defense Bioscience Review Vol. II, 923-934.
31. Fidder, A., Noort, D., De Jong, L.P.A., and Benschop, H.P. (2000) Arch. Toxicol., in press.
32. Noort, D., Hulst, A.G., Fidder, A., De Jong, L.P.A., and Benschop, H.P. (1999) Chem. Res. Toxicol., submitted for publication.

KEY WORDS

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