

Development of a Procedure for the Detection of Exposure of Humans and Animals to Organophosphorus Warfare Agents by the Results of Chemical Analysis of Bodily Fluids

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According to Chemical Weapons Convention, Part XI, Appendix 4, e-17 “Samples of importance in the investigation of alleged use include ... biomedical samples from human or animal sources (blood, urine, excreta, tissue etc.)”. Samples are likely would be analyzed off-site by at least two designed laboratories.

Now OPCW Technical Secretariat enlisting help from appropriate laboratories that possess the necessary analytical skills in new techniques for analyzing biological samples. Standard procedures for supporting the OPCW analytical activities in the biomedical field have been undeveloped up to the present time. We are specializing in this area and have developed a number of procedures for identification of biomarkers of organophosphorus warfare agents (OPCWA) in bodily fluids by means of GCMS.

It is now firmly established that the detection in biological samples of isopropyl, pinacolyl, ethyl, or isobutyl esters of methyl phosphonic acid (MPA) provides unambiguous evidence for the exposure of an organism to sarin, soman, VX, or RVX (Russian VX), respectively. MPA is a universal marker of exposure to any of these agents. The determination of alkyl phosphonates by HPLC and capillary electrophoresis has been reported. However, GCMS is considered to provide a more reliable identification of the metabolites of OPCWA in biological media [1]. The mentioned esters are all nonvolatile and can only be analyzed as derivatives. Comparison of various derivatization procedures showed that the most suitable derivatives for the GCMS analysis of the target compounds in biological matrices are *tert*-butyldimethylsilyl esters. In blood plasma, such derivatives were prepared in a deproteinized dry residue, while in urea, by solid-phase microextraction (SPME) by immersion of the microfiber directly into the sample. The most sensitive and reproducible GCMS analysis of *tert*-butyldimethylsilyl esters of OPCWA metabolites in biological media is reached in the electron impact (EI) mode.

Determination of OPCWA metabolites in blood plasma

Acetonitrile, 1 ml, was added to 1 ml of plasma, the mixture was vigorously shaken and centrifuged for 12 min at 1200 g. The deproteinized plasma was evaporated to dryness on a rotary evaporator at 85°C, silylated with a mixture of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide and dimethylformamide (1:1), and analyzed by GC-EIMS.

Determination of OPCWA metabolites in urine.

Hydrochloric acid, 50 µl of a 6 M solution, was added to 1 ml of urine, the mixture was vigorously shaken and centrifuged for 15 min at 12000 g. The supernatant was decanted to a 4-ml GC vial containing 1 ml of saturated NaCl. A 50/30 DVB/Carboxen/PDMS StableFlex microfiber was conditioned for 15 min in a GC injector at 280°C, exposed over the silylating agent for 5 min in a hermetically sealed vial, immersed into a stirred urine sample for 30 min, and then exposed over the silylating agent for 15 min to derivatize the target analytes absorbed in the microfiber. All operations were performed at room temperature.

Thermodesorption of the derivatives from the microfiber was performed in the GC injector at 250°C in the splitless mode (1 min).

GCMS analysis of both plasma and urine extracts was performed on a Shimadzu QP5000 instrument. The GC system was fitted with a Supelco DB-5 capillary column (25m x 0.2 mm x 33 µm). Helium at 1 ml/min was used as the carrier gas. The oven temperature was programmed to increase from 40(1 min) to 270°C at 5 °C /min. The injector and mass spectrometer transfer line temperatures were 250 and 280°C, respectively. Electron impact ionization was used, electron ionizing energy 70 eV. The mass chromatograms were collected over the range *m/z* range 73 – 400 (SCAN mode) or by selective ion monitoring (SIM mode) at *m/z* 153 and 267.

The most difficult stage of treatment of urine samples is their desalination. The desalination procedure inevitably involves losses of the target analytes because of their high polarity and low molecular weight. The high salt content of the matrix does not hinder SPME analysis. Moreover, samples are deliberately saturated with inorganic salts for better extraction of the analytes from the matrix. Tiong *et al.* have reported a facile procedure for the determination of MPA acid esters in aqueous media, including urine [2]. We successfully applied a similar procedure for the analysis of OPCWA metabolites. In the development of the SPME procedure, an acetonitrile solution of MPA acid esters (*c* 0.5 – 25 µg/ml was introduced into 1ml of distilled

water acidified to pH 1 with HCl or in 1 ml of blank urine. The work on optimization of the analytical procedure for urine is in progress.

Thus we showed that SPME holds promise for extraction of the target analytes from bodily fluids. This technique requires no solvents and complex instrumentation, is labor-consuming, can be easily combined with chromatographic analysis, and is quite suitable for concentrating nonvolatile urine components.

Application of the developed procedures in experiments *in vivo*

The samples were taken from rats exposed to varied doses of nerve agents via intravenous injection. Blood samples were taken 6-30 min after injection, and urine samples, a day after injection.

The key problem to be solved in the analysis of OPCWA metabolites in biological media is their reliable identification for the establishment of exposure of an organism to a specific agent. Quantitative assessment of metabolites is of secondary importance in view of the fact that their measured content in blood plasma or urine cannot provide reliable information of degree of exposure. At the same time, quantitative characteristics of a procedure (detection limit, analyte recoveries, linear range, etc.) are important to establish in the stage of development of the procedure. For quantification of sarin, soman, and RVX metabolites in the experiments *in vivo* we used as internal reference ethyl MPA, a metabolite of American VX. Initially, high doses of agents were used for animal poisoning. This made impossible toxicokinetic studies, but ensured high concentrations of metabolites in body fluids and thus allowed GCMS analysis both in the SCAN and SIM modes. In the first case, metabolites could be identified both by full mass spectra and detected from the mass chromatograms reconstructed by selected ions. It was found that the analysis based on SIM mass chromatograms provides overestimated (higher than theoretically expected) results and fails to ensure reliable identification. The SCAN mass chromatograms with appropriate background subtraction allow more reliable quantification, but the detection limit therewith is decreased by an order of magnitude. However, in view of the priority of reliable identification over sensitivity, we consider the SCAN mode more justified in this specific case.

The results of determination of OPCWA metabolites in rat blood plasma and urine in experiments *in vivo* are presented in Tables 1 and 2, respectively.

Table 1. Results of OPCWA metabolites determination in rat blood plasma in experiments *in vivo* (GCMS in the SCAN mode, identification by full mass spectra)*

Agent	Dose <i>in vivo</i> , mg/kg	Number of LD50, mg/kg	Metabolite	Theoretically expected content, µg/ml	Found, µg/ml
Soman	0.45	5	Pinacolyl MPA	≤2.0	1.8
			MPA		0.4
Sarin	1.2	8	Isopropyl MPA	≤3.2	0.26
			MPA		0.1
RVX	0.09	5	Isobutyl MPA	≤1.3	0.06
			MPA		0.16

* Detection limit, µg/ml: pinacolyl MPA 0.1, isopropyl MPA 0.1, isobutyl MPA 0.1, and MPA 0.5.

Table 2. Results of OPCWA metabolites determination in rat urine SPME-GCMS (SCAN mode)*

Agent	Dose <i>in vivo</i> , mg/kg	Metabolite	Theoretically expected content, µg/ml	Found, µg/ml
Soman	0.06	Pinacolyl MPA	4	1.8
		MPA		1.4
Soman (diluted with blank urine to 1 :10)		Pinacolyl MPA	0.4	0.07
		MPA		0.08
Sarin	0.12	Isopropyl MPA	7.8	2.8
		MPA		0.3
RVX	0.015	Isobutyl MPA	0.6	0.09
		MPA		0.2

Detection limit, µg/ml: pinacolyl MPA - 0.02, isopropyl MPA - 0.2, isobutyl MPA - 0.2, and MPA - 0.2.

As seen from Table 2, SPME-GCMS provides the best results with pinacolyl MPA (soman metabolite).

Owing to their low molecular weight and water solubility, MPA and its esters are excreted with urine in a few days after exposure. In our hands, SPME - GCMS is best suited for the determination of pinacolyl MPA in urine. The greatest advantage of SPME is that it requires no sample preparation. Alkyl methylphosphonates are determined after *tert*-butyldimethylsilylation directly in urine. The detection limit pinacolyl MPA is 0.02 ppm.

REFERENCES

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