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Development and validation of a sensitive gas chromatography–ammonia chemical ionization mass spectrometry method for the determination of tabun enantiomers in hemolysed blood and plasma of different species☆,☆☆

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ABSTRACT

The aim of this study was to develop and validate a fast, sensitive and easily applicable GC–MS assay for the chiral quantification of the highly toxic organophosphorus compound tabun (O-ethyl-N,N-dimethylphosphoramidocyanidate, GA) in hemolysed swine blood for further use in toxicokinetic and toxicodynamic studies. These requirements were fulfilled best by a GC–MS assay with positive chemical ionization with ammonia (GC–PCI-MS). Separation was carried out on a β-cyclodextrin capillary column (Supelco BetaDex® 225) after reversed phase (C18) solid-phase extraction. The limit of detection was 1 pg/ml for each enantiomer (approximately 500 fg on column) and the limit of quantification 5 pg/ml. The GC–PCI-MS method was applied for the quantification of tabun enantiomers in spiked swine blood after hemolysis and in spiked plasma of different species including humans.

1. Introduction

Highly toxic derivates of methylphosphonic and phosphoric acids, such as sarin, cyclosarin, soman, VX and tabun (O-ethyl-N,N-dimethylphosphoramidocyanidate, GA; Fig. 1) are representatives of the most important group of chemical warfare agents (nerve agents). Organophosphorus (OP) nerve agents include an asymmetrical P-atom and consist of at least two stereoisomers. The (+)-P and (−)-P stereoisomers exhibit different toxicological characteristics. (−)-P isomers of sarin, cyclosarin, soman, VX and tabun are much more toxic compared to (+)-P isomers [1]. Determination of toxicological and toxicokinetic parameters of nerve agents for the development of antidotal therapies demands consideration of different biochemical and physiological characteristics of its stereoisomers. Hence, in order to assess toxicokinetic and toxicodynamic properties, reliable analytical methods are required for the specific and selective determination of individual isomers of OP in blood and plasma [1,2].

Analytical methods for the determination of tabun residues in biological media have mainly been established for retrospective detection of exposure. Due to its chemical reactivity the intact parent compound is present in the organism for a few hours and therefore has limited utility for verification analysis [3]. So far, no fully validated methods have been published that can quantify authentic tabun in biological matrices. Instead, a fluoride reactivation assay of the tabun-inhibited butyrylcholinesterase with consequent formation, detection and quantification of fluorotabun (O-ethyl-N,N-dimethylphosphoramidofluoridate) was applied [4–6]. This technique allows a retrospective detection after several weeks. Recently, LC–MS assays have been introduced which target tabun adducted to peptides after enzymatic cleavage of tabun-phosphylated butyrylcholinesterase [7] or albumin [8], prolonging the possible retrospective detection to several months.

Although all these methods are valuable in the verification analysis of tabun exposure, they cannot be applied to the intended toxicokinetic and toxicodynamic studies, as they do not yield information of the original amounts of (+)- and (−)-tabun. The primary metabolites after enzymatic hydrolysis of tabun, i.e. O-ethyl-N,N-dimethylphosphoramidic acid and O-ethyl-cyanophosphoric acid, are far too unstable to be used for detection and quantification purposes, whereas the secondary metabolite, O-ethyl-phosphoric acid, is an unspecific ubiquitous excretion product [9–12]. Therefore, in order to investigate the chiral enzymatic and non-enzymatic hydrolysis of tabun, an assay is needed that can detect the parent authentic compound.

Various analytical assays for the detection and identification of tabun and its contaminations in different environmental matrices (e.g. aqueous solutions, office media, soil) or in recovered muni-

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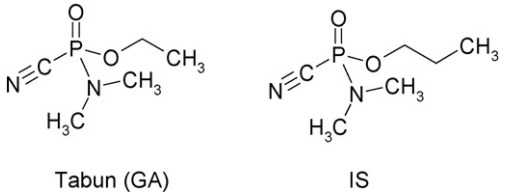

Fig. 1. Chemical structures of tabun (O-ethyl-N,N-dimethylphosphoramidocyanidate, GA) and of O-propyl-N,N-dimethylphosphoramidocyanidate (IS).

tions and munition blocks have been developed, most of them using different GC–MS and LC–MS techniques (e.g. Refs. [13–18]). However, these assays have not been designed for analysis in biological matrices or chiral separation.

To our knowledge, so far only three papers have dealt with the chiral separation of tabun. Degenhardt et al. [19] developed a procedure for the separation and quantification of tabun isomers, using a specially designed europium shift-reagent coated column for GC–MS/NPD (nitrogen phosphorus detector). This assay, however, did not lead to a complete baseline separation of the tabun enantiomers. Van den Berg also separated both enantiomers by ^{1}H NMR semi-quantitatively, using another lanthanide shift-reagent [20]. However, no validation data and information on sample preparation have been reported. Smith et al. presented several alkyl-substituted cyclodextrin stationary phases on which a separation of the tabun enantiomers was possible by GC-AES (atomic emission spectroscopy) but these authors did not report a validated assay for quantification [21]. Furthermore, this group did not examine alkyl-silyl-substituted cyclodextrin derivatives as stationary phases.

The aim of the present study was to develop a thoroughly validated, readily applicable and sensitive GC–MS method for baseline separation and quantitative determination of GA enantiomers in biological matrices at relevant concentrations for toxicokinetic and toxicodynamic studies. We defined this concentration range from 0.005 to 100 ng/ml of each enantiomer.

2. Experimental

2.1. Chemicals and reagents

O-Ethyl-N,N-dimethylphosphoramidocyanidate (GA; for structure see Fig. 1; >98% by ^{1}H NMR and ^{31}P NMR) and the internal standard (IS) O-propyl-N,N-dimethylphosphoramidocyanidate (for structure see Fig. 1; >97% by ^{1}H NMR and ^{31}P NMR) were supplied by the German Ministry of Defence. Ethylenediaminetetraacetic acid tripotassium salt dehydrate (99%) and tris(hydroxymethyl)aminomethane (Trizma® base) were obtained from Sigma–Aldrich Chemie (Taufkirchen, Germany). Isolute C18 (EC) cartridges (octadecyl end-capped sorbent; 100 mg, 10 ml) were obtained from Separtis (Grenzach-Wyhlen, Germany). Ammonia (6.0) and methane (5.5) were obtained from Linde (Unterschleißheim, Germany). Helium (6.0) was obtained from Air Liquide Germany (Düsseldorf, Germany). Chloroform (HPLC grade) was obtained from VWR (Darmstadt, Germany). Isopropanol, methanol, hexane (SupraSolv, for gas chromatography) and all other chemicals (analytical grade) and liquid reagents (HPLC grade) were obtained from Merck (Darmstadt, Germany).

2.2. Biosamples

Heparinized swine whole blood was obtained from the local slaughterhouse and was transferred on ice to the lab for further processing. Heparinized rat and guinea pig plasma was purchased from Charles River (Sulzfeld, Germany). Heparinized human plasma was obtained from a local bloodbank. Swine blank blood as well as pooled rat, guinea pig and human blank plasma samples were used for the development, validation and application of the procedure.

2.3. Sample preparation and extraction procedure

As our goal was to cover a tabun concentration range from 0.005 to 100 ng/ml of each enantiomer, two different sample preparation procedures were applied. Tabun concentration range I reached from nominal sample concentrations of 0.005–2.5 ng/ml of each enantiomer, tabun concentration range II reached from nominal sample concentrations of 0.5–100 ng/ml of each enantiomer (see Table 1). The concentration ranges overlapped for practical reasons.

Satisfactory stabilization of tabun was achieved by successive treatment of EDTA-swine blood samples with two formate buffers. The blood sample (1.0 ml) was hemolysed with 3 ml 50 mM sodium formate buffer pH 3.75. After 1 min, 2 ml 100 mM sodium formate buffer pH 3.75 was added. The resulting mixture was centrifuged at 4000 rpm (Rotina 35 r, Hettich Zentrifugen, Tuttlingen, Germany) and 4 °C for 5 min. After the addition of 0.05 ml IS solution (4 ng/ml O-propyl-N,N-dimethylphosphoramidocyanidate in isopropanol) the samples were mixed (15 s) on a rotary shaker and loaded on SPE cartridges previously conditioned with 1 ml methanol and 2.5 ml deionized water. After loading, the cartridges were washed with 2 ml deionized water. Vacuum was applied until the cartridges were dry, and the analytes were eluted with 1 ml chloroform into 5 ml glass tubes. The eluate was evaporated to a final volume of about 50 μl in a TurboVap LV workstation (Caliper Life Sciences, Rüsselsheim, Germany) under a gentle stream of nitrogen (5 psi) at 30 °C. The residue was transferred to an autosampler vial with a glass insert for analysis. 40 μl of this solution were injected into the GC–MS system, thereby covering tabun concentration range I (see Table 1). For tabun concentration range II (see Table 1), samples were prepared as described above except for the IS concentration (0.05 ml of 200 ng/ml). The SPE eluate was transferred into autosampler vials without glass inserts. Aliquots (5 μl) of this solution were injected into the GC–MS system.

2.4. GC–MS conditions

The studied analytes were quantified in hemolysed swine blood using an Agilent Technologies (Waldbronn, Germany) HP 6890N gas chromatographic system and a 5975N MS detector with ammonia positive ion chemical ionization mass spectrometry (GC–PCI-MS). The system was equipped with a cold injection system CIS 4plus (Gerstel, Mülheim an der Ruhr, Germany). Chromatographic separation was performed on a Supelco BetaDex® 225 column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) from Sigma–Aldrich Chemie (Taufkirchen, Germany). Helium carrier gas was set at a constant flow of 1.3 ml/min. A solvent vent stop-flow injection mode was used. An aliquot of 40 μl was injected into an unpacked deactivated baffled siltek liner within 2.0 min at a pre-column pressure of 0 bar. For the determination of the injection parameters a large volume injection (LVI) calculator programme (Gerstel) was used. The injector initial temperature was 50 °C. The final temperature of 200 °C was reached at a rate of 12 °C/s and was kept constant for 2.0 min. The initial time and vent time were set at 2.10 and 2.08 min, respectively. The vent flow rate was set at 10 ml/min, the purge flow rate at 50 ml/min with a purge time of 4.08 min. The column temperature program started at 50 °C which was held for 4.4 min. Then the temperature was raised to 170 °C with a rate of 12 °C/min and was maintained for 5 min. In the case of tabun concentration range II (see Table 1) the injection volume was reduced to 5 μl, with an injection time of 0.3 min. The initial time and vent time were set at 0.35 and 0.33 min, respectively. The vent flow rate was set at 10 ml/min, the purge flow rate at 50 ml/min