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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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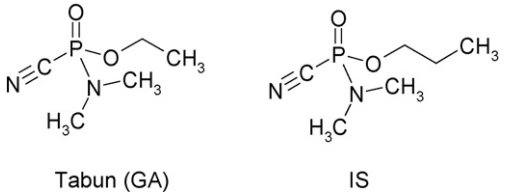

Fig. 1. Chemical structures of tabun (O-ethyl-N,N-dimethylphosphoramidocyanidate, GA) and of O-propyl-N,N-dimethylphosphoramidocyanidate (IS).

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

Table 1
Validation data of the GC–PCI-MS (ammonia) method for the tabun enantiomers in two concentration ranges (matrix: hemolysed blood).

Tabun enantiomer	LOD (pg/ml)	Linearity range (ng/ml)	R^2	Nominal concentration (ng/ml)	Bias[a] (%)	Precision (RSD, %)		Recovery (mean ± SD, %)
		Range I				Intra-assay	Inter-assay	
GA (+)	1	0.005–2.50	0.987	Low 0.01	5.8	8.8	15.6	67.2 ± 6.6
				Medium 0.25	5.2	6.1	9.1	
				High 2.50	−1.7	6.8	10.4	67.5 ± 6.9
GA (−)	1	0.005–2.50	0.988	Low 0.01	8.5	7.1	13.4	65.0 ± 5.5
				Medium 0.25	1.5	7.5	8.1	
				High 2.50	−2.6	8.7	8.1	65.3 ± 5.6
Tabun enantiomer	LOD (ng/ml)	Linearity range (ng/ml)	R^2	Nominal concentration (ng/ml)	Bias[a] (%)	Precision (RSD, %)		Recovery (mean ± SD, %)
		Range II				Intra-assay	Inter-assay	
GA (+)	<0.5	0.5–100.0	0.994	Low 1.0	−2.2	5.7	5.1	70.5 ± 4.2
				Medium 10.0	6.3	6.3	7.2	
				High 100.0	0.7	3.3	8.6	72.6 ± 5.4
GA (−)	<0.5	0.5–100.0	0.995	Low 1.0	−1.9	5.2	5.0	70.8 ± 4.9
				Medium 10.0	2.0	6.6	7.2	
				High 100.0	0.8	3.5	8.4	73.1 ± 4.3

[a] Bias = [(mean calculated concentration − nominal concentration)/nominal concentration)] × 100.

with a purge time of 2.60 min. The column temperature program started at 50 °C which was held for 3.0 min. Then the temperature was raised to 170 °C with a rate of 12 °C/min and was maintained for 5 min.

The following conditions were used for mass spectrometric analysis: positive chemical ionization with ammonia, ammonia flow rate of 2.0 ml/min, transfer line temperature 230 °C, ion source temperature 210 °C, solvent delay 12 min. The target ions $[M+NH_4]^+$ were detected in the selected ion monitoring (SIM) mode (dwell time 125 ms each) at m/z 180 for the tabun isomers and at m/z 194 for the IS. Quantification was carried out using weighted least squares ($1/\chi^2$) calibration curves which were constructed by plotting the peak area ratios of analyte to IS.

2.5. Method validation

2.5.1. Preparation of stock and spiking solutions and testing of selectivity

Stock solutions of racemic tabun and IS (1.0 mg/ml) were prepared in hexane and stored for 1 month at room temperature. Working solutions of each analyte were prepared daily in isopropanol in volumetric flasks by dilution of the stock solutions.

Method selectivity was tested by analysing blank swine blood samples from 10 different animals and 10 swine blood samples spiked with tabun and IS, respectively.

2.5.2. Linearity of calibration

Aliquots of blank blood samples (1 ml) were processed as described in Section 2.3. Then aliquots of these hemolysed blood samples were spiked with 0.05 ml of the corresponding analytical standard solutions and 0.05 ml of IS solution to obtain calibrators at nine different concentration levels across the range of 0.01–5 ng/ml racemic tabun, resulting in 0.005–2.5 ng/ml (+)- and (−)-tabun. Replicates ($n = 6$) of each concentration were analyzed as described above. Calibration curves were prepared daily with each batch of validation and authentic samples using the same concentrations (single measurements per level). In case of tabun concentration range II, aliquots of the hemolysed blood samples were spiked with 0.05 ml of the corresponding analytical standard solutions and 0.05 ml of the higher concentrated IS solution to obtain calibrators at eight different concentration levels across the range of 0.5–100 ng/ml (+)- and (−)-tabun.

2.5.3. Accuracy and precision

Quality control (QC) samples (low, medium and high; nominal concentrations 0.01, 0.25 and 2.5 ng/ml (+)- and (−)-tabun) were analyzed according to the procedure described above in triplicate on 8 days. For tabun concentration range II (see Table 1), the nominal concentrations were set at 1.0, 10.0 and 100.0 ng/ml. The concentrations of the analytes in the QC samples were calculated by using the daily constructed calibration curves. Accuracy was calculated for each analyte as bias, i.e. as the percentage deviation of the mean of all calculated concentration values at a specific level from the corresponding nominal concentration. The data for intra- and inter-assay precision of the method were calculated as relative standard deviations (RSD) using analysis of variance according to [27].

2.5.4. Bench top stability/processed sample stability studies

For estimation of stability of the analytes in processed samples under the conditions of GC–MS analysis, low and high QC samples ($n = 10$, each) were extracted as described above. The resulting extracts at each concentration level were pooled for stability testing over 36 h. Aliquots of these pooled extracts were transferred to autosampler vials and injected under the conditions of a routine analytical run at time intervals of 2 h. Stability of the analytes in

extracts was determined by plotting absolute peak areas of each analyte at each concentration versus injection time. Instability of processed samples would be indicated by a negative slope significantly different from zero ($P \leq 0.05$).

2.5.5. Freeze/thaw and long-term stability studies

For evaluation of freeze/thaw stability, QC samples (low and high) were analyzed prior to (control samples, $n = 6$) and after three freeze/thaw cycles (stability samples, $n = 6$). For each freeze/thaw cycle, the samples were frozen at −80 °C for 21 h, thawed and kept at ambient temperature for 3 h. The experiments were carried out in parallel with the accuracy and precision experiments, and the concentrations of the control and stability samples were calculated by using daily constructed calibration curves. Stability was tested against an acceptance interval of 90–110% for the ratio of the means (stability samples versus control samples) and an acceptance interval of 80–120% from the control samples mean for the 90% confidence interval of stability samples.

The experimental design and procedure for evaluation of long-term stability were similar to those used to examine freeze/thaw stability. Analyte stability for long-term storage was tested by analyzing spiked samples at two concentrations of the analytes (low and high) before (control samples, $n = 6$) and after storage for 1 month at −80 °C (long-term stability samples, $n = 6$).

2.5.6. Detection and quantification limits

For determination of the limit of detection (LOD) of the method in the SIM mode spiked blanks were analyzed. The lower concentrations of the calibration curves were used to determine the limit of quantification (LOQ). Signal-to-noise (S/N) values of 3:1 and 10:1 were considered for LOD and LOQ, respectively.

2.5.7. Recovery

Recovery was determined at low and high concentration levels ($n = 5$ each, concentrations for the respective tabun concentration ranges see Table 1). Isopropanol solutions (0.05 ml) containing GA at concentrations resulting in the low and HIGH plasma concentration levels in plasma, respectively, were spiked to a 6 ml mixture of blank hemolysed blood and sodium formate buffers. After addition of 0.05 ml of IS, the samples were extracted according to the procedure described above. For control samples ($n = 5$) corresponding to 100% recovery, chloroform solutions of the respective GA and IS concentration levels and processed blank blood samples spiked with GA and IS were used. Recovery was calculated by comparing the peak area ratios (analytes versus IS) of spiked plasma samples and control samples.

2.5.8. Identification of the enantiomers of tabun and the internal standard

For the identification of the tabun enantiomers by means of stereospecific enzymatic hydrolysis pooled human, swine, guinea pig and rat heparinized plasma was diluted 1:15 with 0.1 M TRIS–HCl buffer, pH 7.4, and incubated with 1 μM (±)-tabun for 2 h at pH 7.4 and 37 °C. Details of this assay are discussed in Section 3.5. Analyses were performed by the above-mentioned GC–MS method.

For the enantioselective resolution and identification of IS a HPLC system consisting of a P6890 binary pump, DG-1210 degasser, ASI-100 auto sampler, STH 585 column thermostat, a universal UCI-100 chromatography interface, a Foxy Junior fraction collector (all from Dionex, Idstein, Germany) and a digital Propol polarimeter as a detector with a thermostatted flow-through cell (7 cm length, 1 mm i.d.; Anton Paar Opto Tec, Seelze-Letter, Germany) was used. Chromatographic resolution was achieved on a CHIRACEL OD-H column (250 mm length, 4.6 mm i.d., 5 μm particle size; Daicel Chemical Industries, Japan) at a flow rate of 0.5 ml/min of

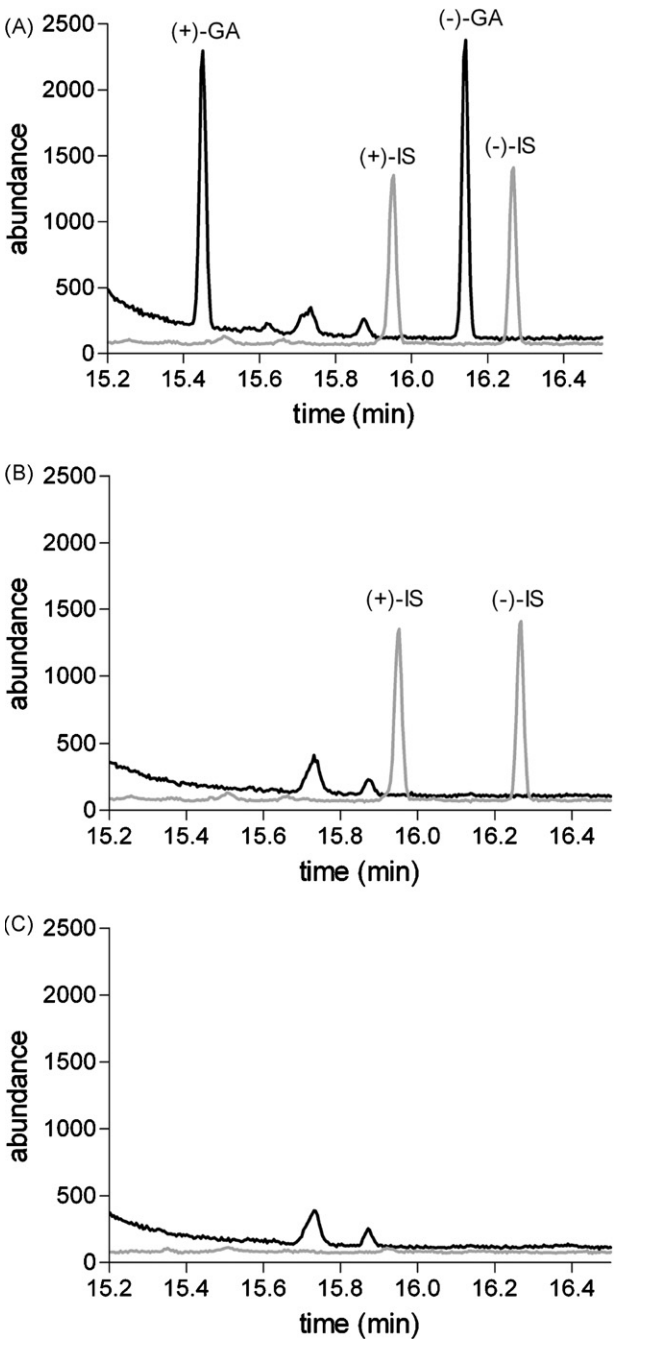

Fig. 2. GC–PCI-MS (ammonia) chromatograms (SIM mode: *m*/*z* 180 for tabun, *m*/*z* 194 for IS) of extracts of a blank swine blood sample spiked to 0.5 ng/ml tabun and 0.2 ng/ml IS (A), of a blank swine blood sample spiked to 0.2 ng/ml IS (B) and of a blank swine blood sample (C).

0.5 vol.% isopropanol in hexane under isocratic conditions by injecting 1.6 mg of racemic IS. The temperature of the column and the flow-through optical cell of the detector were set at 20 °C. Optical rotation was detected at 589 nm (the sodium D line). Then the enantiomers of IS were isolated as two fractions by means of a Foxy Junior fraction collector and injected into the GC–MS for the final identification of the enantiomers.

2.5.9. Proof of applicability

The applicability of the method was tested for the quantification of tabun enantiomers in spiked plasma of different species, i.e. human, swine, rat and guinea pig. For exclusion of possible inter-

ferences, the method was partly re-validated in the investigated systems.

For the proof of applicability the in vitro hydrolysis of tabun enantiomers was determined. Pooled human, swine, guinea pig and rat heparinized plasma was diluted 1:15 with 0.1 M TRIS–HCl buffer, pH 7.4, and incubated with 1 μM (±)-tabun for up to 2 h at pH 7.4 and 37 °C. At different time intervals between 0 and 120 min, aliquots of the incubated plasma samples were taken and processed according to Section 2.3.

The hydrolysis data were analyzed by non-linear regression analysis using GraphPad® Prism 4.0 (GraphPad, San Diego, CA, USA).

3. Results and discussion

3.1. Sample preparation

Tabun is susceptible to hydrolysis in aqueous solutions. The reaction rate can be reduced by lowering the pH value, but hydrolysis cannot be avoided completely [23–27]. At low pH values enzymatic activity of A-esterases in the mammalian blood, spontaneous hydrolysis of some highly toxic O-alkyl methylphosphonofluoridates, inhibition of BChE and AChE by OP and spontaneous reactivation of inhibited cholinesterases are strongly reduced [28–31]. We have previously developed a sample preparation assay for the stabilization of O-cyclohexyl-methylphosphonofluoridate (cyclosarin, GF) [32]. In this method, 2 ml of swine blood are mixed with 6 ml of 50 mM sodium formate buffer pH 3.75 and after 1 min 4 ml of 100 mM sodium formate buffer pH 3.75 are added [32]. This procedure induces hemolysis and reduces blood pH value. This assay was carried out using only half the amounts of the solutions and including a centrifugation step after preparation, resulting in hemolysed blood samples with a final pH value of 4.5. Expectedly, hydrolysis of tabun could not be avoided completely under these conditions. However, when the samples were quickly frozen at −80 °C immediately after asservation, analytes proved to be stable for a period of at least 1 month.

3.2. Extraction procedure

In the early development stages of the presented assay different possible extraction solvents for the SPE were tested. Methanol was not included as tabun is susceptible to degradation by nucleophilic attack of the hydroxyl group in methanolic solutions [17]. Although the use of acetone showed high recovery rates when aqueous solutions of tabun were analyzed, acetone causes blood protein precipitation on the cartridges producing emulsified and difficult to analyze eluates [4,17,33]. The recovery rates when using hexane amounted to remarkably less than 50% [17,33]. Isopropanol, dichloromethane and chloroform allowed acceptable recovery rates. As isopropanol extracts contained remarkably more endogenous substances and dichloromethane was associated with unsatisfying yield after concentration, chloroform was finally used in the optimized LVI method. As shown in Table 1, the mean recovery values ranged between 65 and 73%, with RSD values being below 10% which indicate the suitability of the extraction procedure for extraction of tabun from blood samples.

3.3. GC–MS analysis

For maximum sensitivity of tabun analysis, large volume injection (LVI) techniques have been tested and used. Optimized parameters for the LVI in previous studies included injection volume, type of liner, injection temperature programmes, initial time, vent flow rate, purge flow rate and purge time [34,35]. Physical and chemical properties of tabun and chloroform, as well as the injected sample volume determine the temperature range during evaporation in the injector. During method development temperatures higher than 70 °C led to loss of sensitivity, whereas temperatures below 30 °C are not feasible because of instrumental limitations. Furthermore, in spite of an adequate solvent delay and stop-flow mode, larger injection volumes led to a shortened life time of the filament and of the capillary column. As these limitations did not allow the injection of the whole SPE eluate volume, a combination of LVI and a pre-concentration of the eluate by evaporation were used. Injection volumes of 5, 10, 20, 30 and 40 μl of chloroform solutions and blood extracts in combination with evaporation were tested. Finally, an injection volume of 40 μl was chosen for the tabun concentration range I. For these reasons, the SPE eluate volume was reduced to about 50 μl, and 40-μl aliquots thereof were injected into the GC–MS instrument. For tabun concentration range II an injection volume of 5 μl was applied.

Various chiral GC capillary columns were successfully tested for the baseline separation of the tabun isomers in the early development of the analytical method. In that respect, alkyl-silyl-substituted cyclodextrin stationary phases proved to be less susceptible to column bleeding than alkyl-substituted phases. BETADEX®225 columns were finally selected, since this column enabled the most rapid baseline separation of tabun and IS enantiomers.

A stable-isotope labelled tabun analog would present an ideal IS for tabun analysis by GC–MS, but such as a material is not commercially available. Fluorotabun (O-ethyl-N,N-dimethylphosphoramidofluoridate) was intentionally excluded, because endogenous fluoride anions may generate fluorotabun and thus contribute to the IS concentration [36,37]. In pilot experiments, we tested different O-alkyl-N,N-dimethylphosphoramidocyanidates and O-ethyl-N,N-dialkylphosphoramidocyanidates. O-Propyl-N,N-dimethylphosphoramidocyanidate proved to best fulfill the requirements for IS and used as IS in the present study.

We investigated the utility of different ionization modes. We preferred the use of PCI-ammonia mode over electron ionization (EI, *m*/*z* 133, 162), negative chemical ionization-ammonia (NCI, *m*/*z* 133, 152), PCI-methane (*m*/*z* 191, 203), and NCI-methane (*m*/*z* 133, 152), because PCI-ammonia yielded the highest sensitivity. The LOD in the EI mode, for example, was approximately 10-fold higher than in the PCI-ammonia mode. Additionally, remarkably more endogenous substances could be masked out in the PCI-ammonia mode than in the EI mode, thereby enhancing selectivity (data not shown). In the PCI-ammonia mode, no qualifiers were chosen because of the very soft ionization which produces almost only a single ion, i.e. $[M+NH_4]^+$.

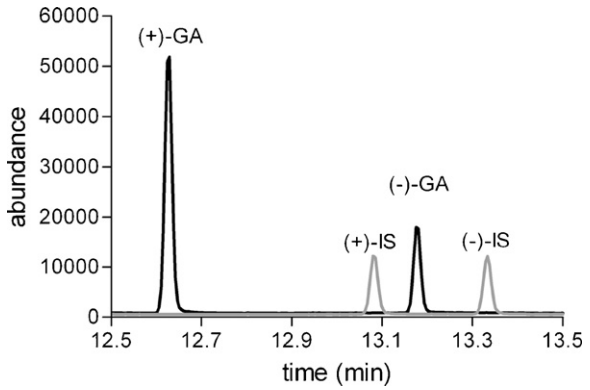

Fig. 3. GC–PCI-MS (ammonia) chromatograms (SIM mode: *m*/*z* 180 for tabun, *m*/*z* 194 for IS) of extracts of a heparinized blank rat plasma sample spiked to 1 μM (162 ng/ml) racemic tabun after incubation of 10 min at pH 7.4 and 37 °C. Differences in retention time compared to Fig. 2 are due to the injection of different samples volumes (5 μl versus 40 μl).

Table 2
Half times of the enzymatic decay of (+)- and (−)-tabun in plasma of different species.

Species	Enantiomer	Half time[a] (min)	95% Confidence interval (min)	R^2
Human	(+)-GA	8.66	8.37–8.98	0.9971
	(−)-GA	8.86	8.49–9.27	0.9957
Swine	(+)-GA	20.2	19.09–21.41	0.9987
	(−)-GA	26.1	24.43–27.97	0.9984
Rat	(+)-GA	14.3	13.75–14.81	0.9969
	(−)-GA	3.71	3.39–4.17	0.9644
Guinea pig	(+)-GA	9.81	9.40–10.27	0.9961
	(−)-GA	22.4	21.07–23.79	0.9914

[a] Half time is given as the mean of three independent assays.

3.4. Method validation and analyte stability

The analytes were stable in processed samples for a period of more than 36 h when stored at room temperature. Expectedly, the stability criteria for the analytes in spiked hemolysed blood samples over three freeze/thaw cycles were not fulfilled. As the assay was developed for use in toxicokinetic and toxicodynamic studies, the limited freeze/thaw stability is considered acceptable. No instability of analytes in spiked plasma samples was observed when stored at −80 °C for a period of 1 month. Thus, analyte concentration in the stability samples was 92 ± 3% of the concentration in the respective control samples.

Neither racemisation nor deracemisation of tabun occurred in organic media during 4 weeks (stock solution in hexan, working solutions in isopropanol, chloroform eluates from spiked blood samples). Also, neither racemisation nor deracemisation of tabun were observed during storage of the concentrated eluate at −80 °C for a 1-month period.

The described procedure was validated according to internationally accepted recommendations [22]. The validation data are summarized in Table 1. Fig. 2 shows no interfering peaks in the extracts of swine blood samples. Intra- and inter-assay precision (RSD) was within the required limits of ≤15%. Accuracy expressed as bias was within the acceptance interval of ±15% of the nominal values [38,39].

3.5. Identification of enantiomers

For the identification of the tabun enantiomers by means of stereoselective enzymatic hydrolysis, 1 μM (±)-tabun was incubated in pooled diluted heparinized plasma from different species (rat, swine, guinea pig, man) for 2 h at pH 7.4 and 37 °C. The use of heparinized plasma instead of EDTA plasma as described in the sample preparation procedure ensured that plasma organophosphate hydrolases were not inactivated and were still able to hydrolyze (±)-tabun [40–42]. Degenhardt et al. reported that (−)-tabun is preferentially hydrolyzed by "phosphorylphosphatases" from rat plasma, resulting in enantiomeric excess (ee) of (+)-tabun up to 100%, albeit in low yield. On the other hand, "phosphorylphosphatases" from swine, guinea pig and human plasma showed only a slight preference for the hydrolysis of (+)-tabun, resulting in a slight enrichment of the (−)-enantiomer [19]. According to these findings the later eluting tabun peak of the present assay was assigned to (−)-tabun. Fig. 3 shows a chromatogram from the analysis of (+)- and (−)-tabun in rat plasma after 10 min incubation of (±)-tabun.

The identification of the IS enantiomers was carried out as described elsewhere [2]. Different hexane–alcohol mobile phases were tested in semi-preparative experiments. A final elution of 0.5 vol.% isopropanol in hexane was found to be optimal satisfactory chromatographic resolution, enabling the isolation of the individual IS enantiomers with an ee of 99.8% each. The IS peak with the smaller retention time in the present method was identified as (+)-IS.

3.6. Proof of method applicability

Spiked plasma samples of humans, swine, rats and guinea pigs were analyzed by the presented method. No remarkable differ-

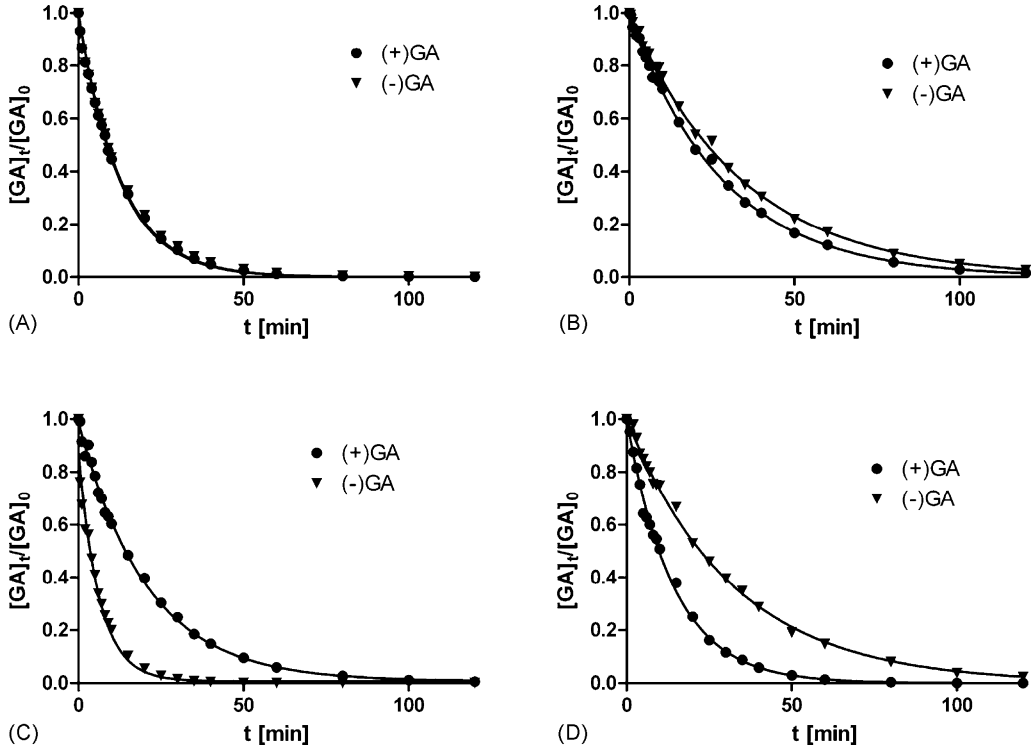

Fig. 4. Decay of (+)- and (−)-tabun in heparinized blank plasma samples diluted 1:15 with 0.1 M TRIS–HCl buffer, pH 7.4, and spiked to 1 μM (162 ng/ml) racemic tabun at pH 7.4 and 37 °C. Data are given as means of three repetitive assays. A, human; B, swine; C, rat; D, guinea pig.

ences between the respectively acquired mass chromatograms were observed, with regard to possible matrix interferences.

The enzymatic decay of tabun in plasma (see Section 2.5.9) was measured over a time period of 2 h and the results obtained are given in Table 2 and Fig. 4. Hereby, no stereoselective hydrolysis of tabun was recorded in human plasma and only a slight difference was observed in pig plasma. In contrast, (+)-GA was hydrolyzed substantially faster than (−)-GA in guinea pig plasma while a reversed pattern was found with rat plasma. The reaction rate of tabun hydrolysis in plasma was dependent on the species. Thus, tabun degradation proceeded markedly faster in human and rat plasma than in guinea pig and swine plasma.

4. Conclusions

The GC–PCI-MS (ammonia) method presented here allows fast, reliable and chiral separation and quantification of tabun enantiomers in hemolysed blood and plasma of different species. The method was validated and fulfils main validation criteria for accuracy and precision. The LOD and LOQ values are 1 pg/ml (approximately 500 fg on column) and 5 pg/ml, respectively. This method proved to be useful for quantifying the in vitro hydrolysis of tabun enantiomers in human, swine, guinea pig and rat plasma.

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