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Chromatographic resolution, characterisation and quantification of VX enantiomers in hemolysed swine blood samples

Georg Reiter[a,*], John Mikler[b], Ira Hill[b], Kendal Weatherby[b], Horst Thiermann[a], Franz Worek[a]

[a] Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, 80937 Munich, Germany
[b] Casualty Management Section, Defence Research & Development Canada Suffield, PO Box 4000 Stn Main, Medicine Hat, Alberta, T1A 8K6 Canada

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ABSTRACT

The present study was initiated to develop a sensitive and highly selective method for the analysis of the enantiomers of the nerve agent VX (*O*-ethyl *S*-[2(diisopropylamino)ethyl] methylphosphonothioate) in blood samples for toxicokinetic and therapeutic research. To achieve this goal, analytical and semi-preparative enantioseparation of VX were carried out with gas and liquid chromatography. The GC chiral stationary phase was HYDRODEX-β-TBDAc (beta cyclodextrin), on which VX was baseline-resolved. On the chiral HPLC phase CHIRALCEL OD-H the enantiomers of VX were isolated with enantiomeric excess >99.99%. They were characterised by specific optical rotation (±25.8 deg ml dm^{-1} g^{-1} at 20 °C and 589 nm) and by determination of cholinesterase inhibition rate constants. For the quantitative chiral detection of VX the enantioresolution was realized on the HPLC chiral phase CHIRAL AGP. A specific procedure was developed to isolate VX from swine blood samples thereby stabilising its enantiomers. The limit of detection was 200 fg per enantiomer on column. The absolute recovery of the overall sample preparation procedure was 75%. After an intravenous and percutaneous administration of a supralethal dose of VX in anesthetised swine (+)-VX and (−)-VX could be quantified up to 720 min.

1. Introduction

Highly toxic derivates of methylphosphonic and phosphoric acids such as sarin, soman, tabun and VX 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 substantially different toxicological characteristics. (−)-P isomers of sarin, soman, tabun and VX are much more toxic compared to (+)-P isomers [1]. The determination of toxicological and toxicokinetic parameters of nerve agents for the development of antidotal therapies requires consideration of different biochemical and physiological characteristics of its stereoisomers. Therefore, to assess toxicokinetic and toxicodynamic properties reliable analytical methods are required for the specific and selective determination of individual isomers of free OP in the blood [1–3].

Methods for the quantitative chiral detection of the nerve agent VX in biological matrices were not developed up to now. One reason for this is the difficulty of separating and quantifying of VX enantiomers at relevant *in vivo* concentrations by means of GC and LC; resultantly to date VX isomers had not been separated with GC. The enantioresolution of VX with LC (HPLC) methods has been carried out by several research groups but despite these efforts a quantitative method on the basis of the chiral resolution of VX by LC has yet to be developed [4–6]. Hence, only the toxicokinetics of racemic VX has been investigated in guinea pig and marmoset studies [6].

* Corresponding author. Fax: +49 89 3168 2333.
E-mail address: georgreiter@bundeswehr.org (G. Reiter).

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The only possibility for the quantitative toxicological investigation of VX isomers was stereospecific synthesis. The first synthesis and determination of the absolute configuration and toxicological characterisation of VX enantiomers were carried out by scientists at the Chemical Defence Establishment in Porton Down [7].

The present study was initiated to develop a procedure for the quantitative determination of VX isomers in biological matrices for toxicological and therapeutic investigations. The enantioseparation of VX was carried out with gas and liquid chromatography and an optimized LC–MS/MS method was validated for the chiral quantification of VX in blood samples. The procedure was applied to the detection of VX in hemolysed swine blood samples obtained after an intravenous or percutaneous administration of 2× and 3× LD_{50} VX, respectively.

2. Experimental

2.1. Materials

The following materials, GC and HPLC columns were obtained commercially:

- HYDRODEX-β-TBDAc (25 m length, 0.25 mm i.d., 0.2 μm film thickness) from Macherey–Nagel (Düren, Germany).
- CHIRAL AGP (150 mm length, 2 mm i.d., 5 μm particle size, Regis Technologies, USA) from Thermo Fisher Scientific (Dreieich, Germany).
- CHIRALCEL OD-H (250 mm length, 4.6 mm i.d., 5 μm particle size) from Daicel Chemical Industries (Japan).
- Deionised water (Tracepur, for organic trace analysis), *n*-hexane (UniSolv, for organic trace analysis), 2-propanol and methanol (SupraSolv, for gas chromatography), acetonitrile (LiChrosolv, hypergrade for LC–MS), formic acid (98–100%, analar), aqueous ammonia (25%, aristar) from Merck (Darmstadt, Germany).
- Isolated human serum butyrylcholinesterase (BChE; E.C. 3.1.1.8), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCh), *S*-butyrylthiocholine iodide (BTCh) from SIGMA–ALDRICH Chemie (Taufkirchen, Germany).
- Sodium hydroxide (99.99%, Aldrich), tetradecane (99.5%, for gas chromatography, Fluka) from SIGMA–ALDRICH Chemie (Taufkirchen, Germany).
- Sodium heparin (5000 U/ml in aqua) from Ratiopharm (Ulm, Germany).
- EDTA tubes from Becton Dickinson (New Jersey, USA).
- Sodium pentobarbital (Bimeda-MTC, Cambridge, Ontario, Canada).

O-Ethyl *S*-[2(diisopropylamino)ethyl] methylphosphonothioate (VX) and the internal standard (IS) *O*-isobutyl *S*-[2(diisopropylamino)ethyl] methylphosphonothioate (>98% by GC–MS, ^{1}H NMR and ^{31}P NMR) were supplied by the German Ministry of Defence, Bonn. VX of same quality for *in vivo* experiments was synthesized at the Canadian National Single Small Scale Facility at DRDC Suffield.

For the preparation of hemoglobin-free erythrocyte ghosts human blood samples were provided by volunteers and pig blood samples were gathered at the local abattoir using sodium heparin (30 U/ml) as anti-coagulant (Section 2.9).

2.2. Standards and spiking solutions

Stock solutions of VX and IS (1%, w/v) for analytical experiments were prepared in hexane and stored for up to 1 month at −20 °C in glass vials. The stock solutions were further diluted in acetonitrile prior to final dilution in deionized water (spiking solution) on the day of the experiment. In blood samples, the volume of spiking solution did not exceed 1% (v/v) and the final concentration of acetonitrile was less than 1%. The solution of 5% VX in hexane for enantioselective, semi-preparative HPLC (cf. Section 2.8) was prepared on the day of the experiment.

2.3. Instrumentation

The gas chromatographic system 6890N with a 5975N MS detector (GC–EI-MS, Agilent Technologies, Waldbronn, Germany) was used for the gas chromatographic resolution of VX enantiomers. The system was equipped with a cold injection system CIS 4^{Plus} (Gerstel, Mülheim an der Ruhr, Germany).

The analytical LC–MS/MS system for the quantification of VX enantiomers consisted of two binary LC-20AB pumps (pump 1 and pump 2, Fig. 1), a LC-20AD pump (pump 3), a DGU-20A3 degasser (degasser 1), a DGU-20A5 degasser (degasser 2), SIL-20AC auto sampler, CTO-20A column thermostat with two 6-port switching valves, a CBM-20A communication bus module (all from Shimadzu, Duisburg, Germany) and a triple quadrupole 4000 Q Trap mass spectrometer as detector (Applied Biosystems, Darmstadt, Germany).

The semi-preparative isolation of VX enantiomers was performed with an HPLC system consisting of a P680 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 automatic 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).

A Turbo Vap LV workstation (from Caliper Life Sciences, Rüsselsheim, Germany) and a 5810R centrifuge (Eppendorf, Hamburg, Germany) were used for sample preparation.

Enzyme assays were run on a Cary 3Bio spectrophotometer (from Varian, Darmstadt, Germany).

A Siemens SC 7000 patient monitor (Siemens, MA, USA) was used for the continuous monitoring of physiological parameters of the anaesthetised animals (domestic swine, cf. Section 2.5).

A ventilator Hallowell EMC Model 3000 (Pittsfield, MA, USA) was used for assisted ventilation of the VX exposed animals.

2.4. Laboratory animals, in vivo experiments and blood sampling

2.4.1. Domestic swine experiments

Castrated male York–Landrace cross pigs (~20 kg) were purchased from a local supplier and housed indoors in the DRDC Suffield vivarium. The swine were allowed to acclimatise for at least 1 week prior to experimental use. Animals were fed until the evening prior to surgery and were allowed tap water *ad libitum*. In conducting this research the authors adhered to the "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation" published by the Canadian Council on Animal Care. The animal experiments were approved by the responsible ethics committee at DRDC Suffield. At the end of the experiment all animals were euthanized by intravenous (i.v.) injection of 8 ml (540 mg/ml) sodium pentobarbital.

2.4.2. Anaesthesia

The animals underwent an inhalation induction with 5% isoflurane in a carrier gas of 100% oxygen (O_2) at a flow rate of 8 l min^{-1}. Post-induction, the animals were placed in the dorsal recumbent position on a heated operating table. Core body temperature was maintained at ~38.0 °C. After intubation, the isoflurane concentration was reduced to 3% in 100% O_2 at a flow rate of 2 l min^{-1}. Once

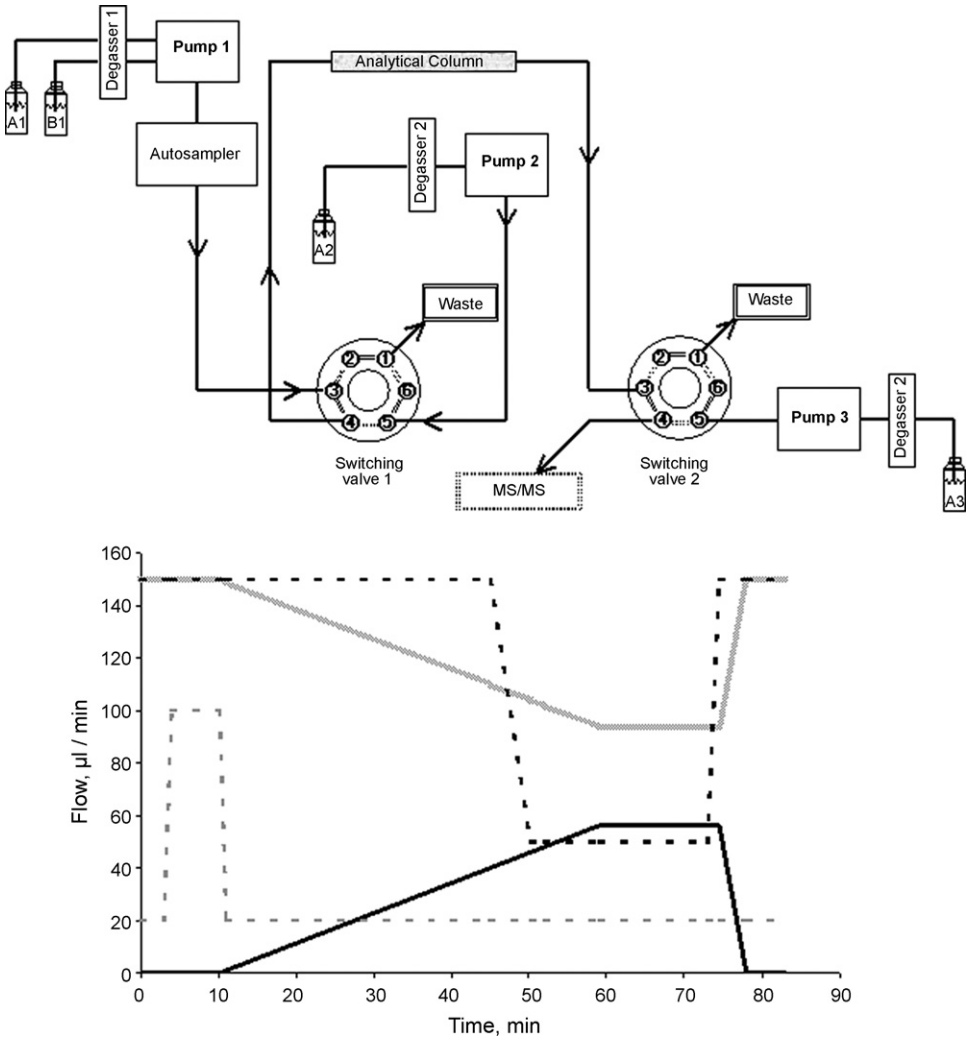

Fig. 1. Schematic diagram of LC–MS/MS with switching valves and flow diagram of the eluents: eluent A1 (solid grey line), eluent B1 (solid black line), eluent A2 (dashed grey line), eluent A3 (dashed black line). Mobile phases A1 and B1 were pumped through the column and into the mass spectrormeter between 45 and 75 min. Mobile phase A3 was pumped into the mass spectrometer from 0 to 45 min and after 75 min.

all monitors and catheters were placed and affixed, isoflurane was maintained at a rate of 2% in room air supplemented with oxygen to a FiO_2 of 0.3. Animals were allowed to stabilize for at least 30 min, during this time steady-state anaesthesia (SSA) was established. The animals received 0.9% normal saline via an i.v. line for fluid replacement and cystostomy was performed in order to monitor urinary output. Continuous physiological parameters were monitored while the animals were under anaesthesia using a Siemens SC 7000 patient monitor. If required following VX exposure, mechanical ventilation with oxygen-supplemented air was provided at a rate of 150–200 ml per cycle at 20 cycles per minute.

2.4.3. Nerve agent administration

A $2\times$ LD_{50} dose of VX, 24.6 μg/kg, was administered as an intravenous (i.v.) injection in a total volume of 3 ml. The nerve agent was diluted in 2-propanol prior to a final dilution in saline immediately prior to administration. The final concentration of 2-propanol was less than 1%. A $3\times$ LD_{50} dose of undiluted VX, 186.9 μg/kg, was pipetted onto a clipped area on the inner surface of the ear pinna.

2.4.4. Medical countermeasures

In i.v. VX poisoned pigs atropine sulphate (AS; 0.03 mg/kg) was injected i.m. at 1, 31 and 61 min post-agent injection. In pigs challenged by p.c. VX atropine was injected i.m. at 30, 180 and 330 min post-exposure. Control animals received AS at 1 min post-agent and then as required based upon a 20% drop in heart rate from SSA. Assisted ventilation was provided as required at a rate 3–4 l/min.

2.4.5. Blood sampling

Blood samples (2 ml) were obtained from an indwelling catheter (20 gauge) placed in a branch of the saphenous artery at the following times post-agent injection 0, 1, 2, 4, 6, 8, 16, 31, 32, 34, 38, 46, 61, 62, 64, 68, 76, 90, 120, 180, 240, 300, 360, 420, 480 min (for i.v. and p.c. application of VX) and additionally 540, 600, 660, 720 min (for i.v. injection of VX). Samples were collected into EDTA tubes and immediately processed. The blood samples were instantly hemolysed, frozen and stored at −80 °C until analysis (cf. Section 2.5). Blood samples from 12 animals were investigated. For experiments with blank and spiked samples individual and pooled hemolysed blood from 12 healthy swine were used. The sample processing for hemolysed blood samples and for *in vivo* samples was identical.

2.5. Sample preparation

Stabilization of VX isomers was achieved by successive treatment of the blood samples with two formate buffers as described

before [3]: 2 ml of blood were mixed with 6.0 ml of 50 mM sodium formate buffer (pH 3.75) and incubated on ice for 1 min before adding 4.0 ml of 100 mM sodium formate buffer (pH 3.75). All samples were immediately frozen in liquid nitrogen and then stored at −80 °C until analysis.

Different protein precipitations, liquid and solid-phase extraction procedures were tested in pilot experiments. The sample processing procedure chosen was the most effective in selectively removing endogenous contaminants in order to minimize disturbance of chromatographic resolution and quantification of VX isomers; this procedure included a combination of protein precipitation and liquid extraction/reconstitution. The samples were thawed and kept in an ice bath until further processed (less than 30 min). Six millilitres of each sample were used for further treatment while the remainder was frozen again to serve as control. Immediately prior to sample preparation the samples were spiked with IS at a final concentration of 100 pg/ml. 192 ml of 2-propanol were added to each sample for protein precipitation. After centrifugation (4000 × *g*, 0 °C, 15 min) the supernatant was transferred to the work station Turbo Vap LV and 2.4 ml of tetradecane was added to each sample. The aqueous organic phase was evaporated at 40 °C with nitrogen (25 psi) up to the remaining tetradecane phase. The extraction/reconstitution was carried out with 1.5 ml deionised water in the same tubes containing the remaining tetradecane and dry endogenous substances. After double centrifugation (8200 × *g*, 10 min at 20 °C and 40 min at 0 °C) of each sample the intermediate phase (∼1.4 ml) was transferred to an autosampler vial for LC–MS/MS analysis.

2.6. GC–EI-MS

The VX enantiomers were base line resolved on a HYDRODEX-β-TBDAc GC column. The elution order and identity of the VX isomers was determined by comparison of chromatograms of racemic VX and the individual VX enantiomers.

GC conditions were as follows:

- Helium as carrier gas at a constant flow of 1.3 ml/min,
- Solvent vent stop-flow injection mode was used. A volume of 5 μl was injected into unpacked, deactivated baffled glass liner within 1 min at a pre-column pressure of 0 bar. The injector initial temperature was 50 °C. The final temperature of 260 °C was reached at a rate of 12 °C/s and was kept constant for 2 min. The initial time was 1.1 min, vent time was 1.01 min, vent flow was 10 ml/min, purge flow was 50 ml/min and purge time was 3 min,
- The column oven temperature program consisted of: 50 °C for 3.5 min, increased to 95 °C at 0.3 °C/min, maintained for 15 min, increased to 115 °C at 0.2 °C/min, maintained for 5 min, increased to 118 °C at 0.2 °C/min and to 180 °C at 10 °C/min to clean the column.

MS conditions for GC analysis of VX were as follows:

- Electron impact ionisation (EI) at 70 eV, transfer line temperature 220 °C, ion source temperature 230 °C, solvent delay 258 min.
- Selected ion monitoring (SIM) mode, dwell-time 100 ms for *m*/*z* 114 and *m*/*z* 127 [8].

2.7. LC–MS/MS analysis

The mobile phases consisted of 25 mM ammonium formate buffer pH 9.0 (eluent A1, Fig. 1), 50 mM ammonium formate buffer pH 9.0—methanol (1:1, v/v, eluent B1), deionised water (eluent A2) and deionised water—methanol (9:1, v/v, eluent A3). Samples (500 μl) were injected and concentrated on the initial sector of an analytical CHIRAL AGP column, followed by a 6.5 min rinse with deionised water (eluent A2) at 100 μl/min (Fig. 1). During injection and water rinse the column served as a "trap" column. The total flow rate of the eluents A1 and B1 through the column was kept at 150 μl/min. The column was thermostatted at 30 °C. The chiral resolution of VX enantiomers was performed with the application of the following linear gradients (Fig. 1): after the rinse of the analytical column eluent B1 was slowly increased from 0% to 37.5% within 49 min, this condition was maintained for the next 15.5 min, then the system was re-equilibrated to 0% eluent B1 within 3.5 min and maintained for 5 min. The elution order and identity of the VX and IS isomers was determined by comparison of chromatograms of racemic VX (IS) and the individual VX (IS) enantiomers.

MS conditions for LC were as follows:

- Positive electrospray ionisation at the following parameters: nitrogen as curtain gas (10 psi), collision gas (setting medium), ion source gas 1 (9 psi) and ion source gas 2 (12 psi), the interface temperature was 400 °C, the ion spray voltage was 5.5 kV, the declustering, entrance, collision cell exit potentials and collision energy were 38, 10, 8 and 30 V, respectively.
- Multiple reaction monitoring (MRM) mode, precursor $[M+H]^+$ → product ion mass transitions were: *m*/*z* 268.4 → 128.2 for VX and *m*/*z* 268.4 → 100.2 for IS [9], dwell-time was 1 s per transition.

2.8. Semi-preparative resolution of VX isomers and determination of the specific optical rotation

The Dionex HPLC system with a digital Propol polarimeter (cf. Section 2.3) were used for the enantioselective resolution of VX. The chromatographic resolution was achieved on a CHIRALCEL OD-H column (cf. Section 2.1) at a flow rate of 600 μl/min of 1% 2-propanol in hexane under isocratic conditions. Different amounts of VX (0.25–5 mg) were injected. The temperature of the column and the flow-through optical cell of the detector were set at 20 °C. Hereby, optical rotation was detected at 589 nm (the sodium D line). Then, the enantiomers of VX were isolated as two fractions by means of a Foxy Junior fraction collector. Finally, the optical rotation of the fractions of isolated VX enantiomers was measured off-line with the Propol polarimeter. The specific optical rotation of the VX enantiomers was evaluated according to [10]. After the optical measurements the VX enantiomers in the fractions were diluted and quantified by LC–MS/MS (cf. Section 2.7). A calibration curve was generated for solutions of racemic VX in water.

2.9. Enzyme assay and determination of inhibition rate constants (k_i) of the VX enantiomers

Acetylcholinesterase (AChE, E.C. 3.1.1.7) and BChE activities were measured spectrophotometrically using a modified Ellman assay [11,12]. Hemoglobin-free erythrocyte ghosts were prepared according to [2] and were used for the determination of the AChE activity. The assay mixture (3.16 ml) contained 0.45 mM ATCh (AChE) or 1.0 mM BTCh (BChE) as substrate and 0.3 mM DTNB as chromogen in 0.1 M sodium phosphate buffer (pH 7.40). Assays were run at 37 °C.

The second order inhibition rate constant (k_i) of racemic VX and the individual VX isomers was determined with human AChE and BChE and pig AChE. Due to large differences in the inhibitory potency of the isomers different procedures were used. For (±)- and (−)-VX a continuous procedure [13] and for (+)-VX a discontinuous procedure [14] were used. The analysis of the data was performed with Prism™ Version 4.00 (GraphPad Software, San Diego, CA, USA).

2.10. Study of specificity

The specificity and stereospecificity of the LC–MS/MS method was checked by analysing six blank hemolysed blood samples and six hemolysed blood samples spiked with VX. All chromatograms were compared with chromatograms from VX standard solutions. *In vivo* samples were also used for additional comparison.

2.11. Determination of recovery rates

The absolute recovery rates were calculated by comparing the chromatogram peak areas of VX in spiked hemolysed blood after the analytical procedure to those of a VX standard solution prepared in water and in blank hemolysed blood matrix (spiked after sample preparation). The recovery was determined at four concentrations (8, 80, 400, 2000 pg/ml; $n = 5$ at each concentration). The recovery compared to IS was determined by comparing peak area ratios obtained from analyte to IS versus the peak area ratios of the same concentration of VX standards and IS spiked in treated hemolysed blood samples. Identical concentrations of VX were used for the determination of the absolute recovery.

2.12. Study of linearity and sensitivity

Linearity test calibration curves were generated within a VX concentration range of 0.5–2000 pg/ml by using spiked pooled hemolysed blood samples ($n = 5$). The linearity of each pair calibration curves was determined by plotting the peak–area ratios $A_{(-)\text{-VX}}/A_{(+)\text{-IS}}$ versus $C_{(-)\text{-VX}}/C_{(+)\text{-IS}}$ and $A_{(+)\text{-VX}}/A_{(+)\text{-IS}}$ versus $C_{(+)\text{-VX}}/C_{(+)\text{-IS}}$ and then using standard linear regression analysis. The limit of detection was determined for mass signals exceeding a signal-to-noise ratio of at least 3:1.

2.13. Accuracy and precision

Accuracy and precision of the analytical procedure was tested at four VX concentrations (2, 20, 200, 2000 pg/ml; $n = 8$, each), in pooled hemolysed swine blood samples.

2.14. VX quantification in in vivo hemolysed blood samples

(−)-VX and (+)-VX concentrations in *in vivo* swine blood samples were determined by LC–MS/MS and were referred to calibration curves of pooled swine blood samples spiked with VX (0.5–2000 pg/ml) and the internal standard (100 pg/ml). Calibration curves were generated for each analytical run.

3. Results and discussion

3.1. Resolution of enantiomers of VX by gas chromatography

The gas-chromatographic separation of VX isomers is a challenging task. Previous attempts, using commercially available chiral GC columns, did not succeed in a separation of VX isomers [4–6]. Here, we present the first chiral separation of VX isomers by GC. The enantiomers of VX were completely (baseline) separated on a HYDRODEX-β-TBDAc column (Fig. 2). The relation of the peak areas of individual isomers of VX remained equal to 1 and was not affected by moderate changes in injector or oven temperature programs (e.g. 240–320 °C for the injector) or by organic solvents in the injection solution (hexane, 2-propanol). This was valid for a concentration range of VX from 20 to 2000 ng/ml in the injection solution. Pilot experiments (data not shown) indicated that VX isomers could be quantified in blood samples by GC–MS.

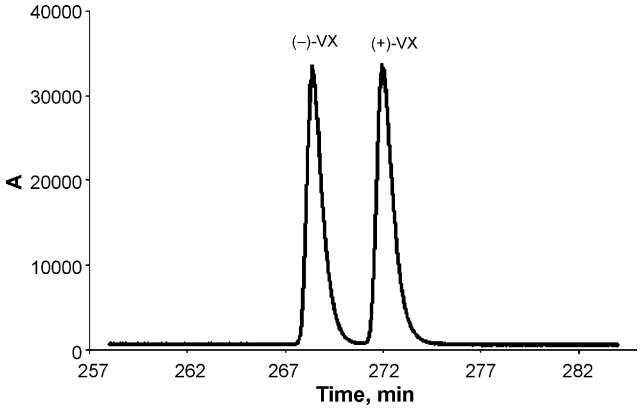

Fig. 2. GC-chromatogram of racemic VX on beta cyclodextrin, HYDRODEX-β-TBDAc column (details of the experimental conditions are provided in Section 2.6).

However, due to the extremely long run time (>280 min) the GC method was considered inadequate for analysis of multiple samples.

3.2. Resolution of enantiomers of VX by HPLC for quantification

LC–MS methods for the quantification of VX enantiomers in blood samples were not previously reported in the open literature. In pilot experiments with different chiral HPLC columns the CHIRAL AGP phase was found to be the most suitable column for the quantification of low concentrations of VX enantiomers (~1–1000 pg/ml) in hemolysed blood samples from VX toxicokinetics studies. The enantiomers of VX and *O*-isobutyl *S*-[2(diisopropylamino)ethyl] methylphosphonothioate (IS) were completely (baseline) separated on a CHIRAL AGP column (Fig. 3).

The relation of the peak areas of individual isomers of VX ($A_{(-)\text{-VX}}/A_{(+)\text{-VX}}$) was equal to 1.10 ± 0.04 after injections of aqueous matrix, 1.11 ± 0.04 from treated spiked pooled blood samples and 1.10 ± 0.05 after injections of eluent A1 for different sample series for a time interval of 4 months. This relation did not vary more than 2% for a series of measurements with LC–MS/MS at a time interval of 2 days at a concentration range of VX from 2 to 5000 pg/ml in the injection solution.

3.3. Semi-preparative resolution of VX by HPLC and characterisation of VX enantiomers

Semi-preparative LC enantioresolution of VX could only be carried out with normal phase chiral chromatography. Using this method the separation of VX enantiomers on a chiral phase CHIRALCEL OD-H was selected [4,5]. Different hexane–alcohol mobile phases were tested in semi-preparative experiments. A final eluent of 1% 2-propanol in hexane, was shown to be optimal for the achievement of chromatographic resolution, enabling the isolation of pure individual VX isomers (Fig. 4). The peak form of both enantiomers was different for all semi-preparative chromatograms, as the peak of (−)-VX was broader and more asymmetric than the (+)-VX peak. Nevertheless, the standard curves of polarimetric detection were identical for both VX isomers (Fig. 4). Approximately 5 mg of each enantiomer were obtained using semi-preparative HPLC on CHIRALCEL OD-H column. The optical antipode in each isolated enantiomeric fraction was not detectable with LC–MS/MS (Fig. 5). The enantiomeric excess (ee) of each separated enantiomer was determined to be >99.99%, based on the enantiomer peak to noise ratio. No racemisation of VX enantiomers in hexane,

2-propanol and water was observed at ambient temperature (cf. Section 3.4).

The specific optical rotation amounted to: -25.8 ± 1.4 for (−)-VX and $+25.8 \pm 0.9$ for (+)-VX (in deg ml dm^{-1} g^{-1}) at 20 °C and 589 nm.

For comparison, literature data of the specific optical rotation are as follows: −12 for (−)-VX (0 °C, from [7]), −30.2 for (−)-VX and +31.0 for (+)-VX (20 °C, from [15]).

The second order inhibition rate constant (k_i) of racemic VX and the individual VX isomers with cholinesterases were determined for human AChE and BChE and pig AChE. The stereoselectivity of AChE and BChE toward the VX enantiomers is characterised by the relationship of two inhibition rate constants $k_{i,(-)\text{-VX}}/k_{i,(+)\text{-VX}}$. A summary of the results is shown in Table 1. The literature data for the stereoselectivity of the cholinesterases toward the VX enantiomers are listed in Table 2 for comparison. The stereoselectivity of BChE is low and substantially lower compared to AChE. Other organophosphates with an asymmetrical central phosphorus atom and thiocholine fragment showed similar characteristics [16].

The experimentally determined inhibitory potency of VX enantiomers with AChE showed large differences, depending on the enzyme source and the purity of the isolated isomers. Hereby, the optical purity of the less reactive (+)-VX isomer is of significant importance, since its inhibition rate constant k_i is strongly affected by potential impurity with (−)-VX. The same applies to the acute toxicity of the biologically less active isomer. In previous studies (cf. Table 2) the purity was examined experimentally with two methods: NMR with chiral shift reagents (semi-quantitative method [17]) and stereospecific synthesis [7]. The precise quantitative determination of the enantiomeric excess was not possible. This was most likely the result of different laboratories using preparations of VX isomers with varying optical purity, primarily (+)-VX, for experiments with the same type of enzyme (e.g. from bovine erythrocytes). Consequently substantially differing values for AChE stereoselectivity were determined (Table 2).

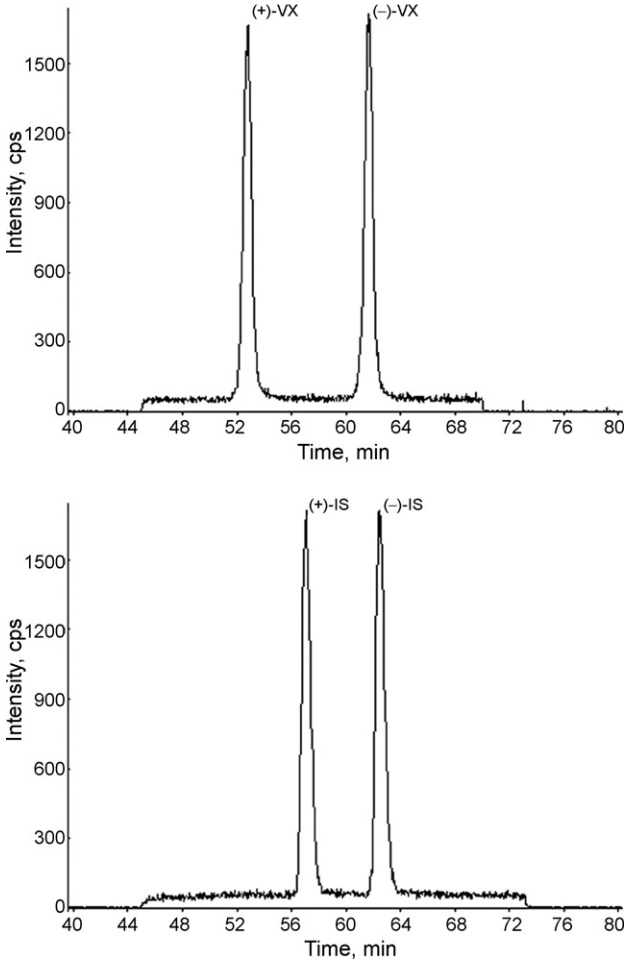

Fig. 3. LC-chromatogram of racemic VX and IS on CHIRAL AGP column (details of the experimental conditions are provided in Section 2.7).

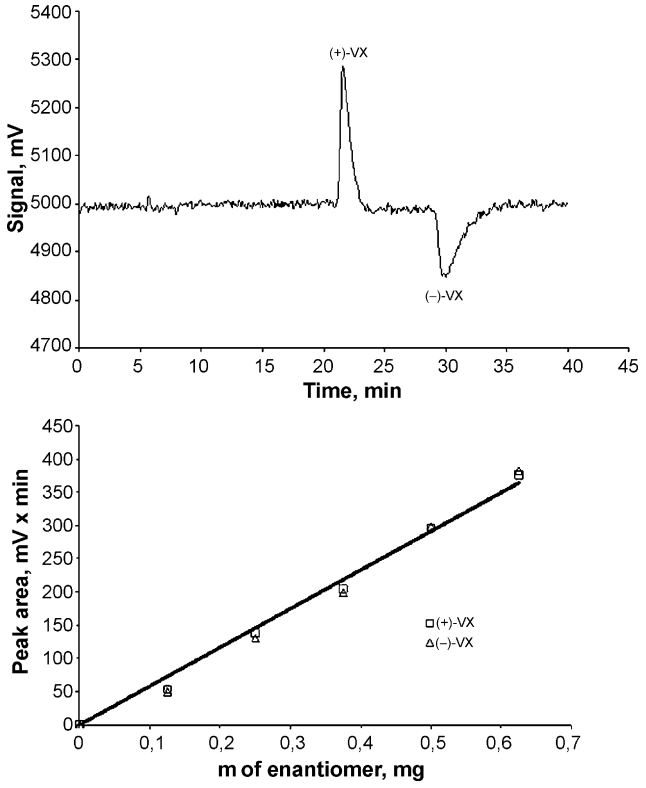

Fig. 4. Chromatogram of racemic VX on CHIRALCEL OD-H column (details of the experimental conditions are given in Section 2.8) and linearity of polarimetric detection of VX enantiomers after the resolution of racemic VX.

3.4. Specificity and stereospecificity of LC–MS/MS assay

The peaks of (−)-VX and (+)-VX isomers were selectively detectable in all standard solutions of racemic VX in water, eluent A1, spiked and *in vivo* hemolysed blood samples. No peaks from endogenous impurities from blood diluted with formate buffers were found in chromatograms of blank pooled blood samples (Fig. 6). Although, the resolution of the VX isomers did not vary in standard, spiked or *in vivo* samples, small variations in retention time were determined for different CHIRAL AGP columns.

Racemisation of VX isomers was investigated using 0.05% solutions of individual enantiomers in hexane, 2-propanol and water. No racemisation occurred in hexane or water solutions of VX enantiomers during storage at ambient temperature or in 2-propanol for up to 16 days and 15 weeks, respectively. Neither racemisation nor deracemisation of VX was observed in treated samples from spiked or *in vivo* blood samples during storage at 4 °C for up to 2 weeks. VX appeared to be stable in stabilized formate buffer solutions at −80 °C for at least 4 months. VX was also stable in samples after two freeze–thaw cycles and incubation in an ice bath for at least 1 h. During the investigation of the

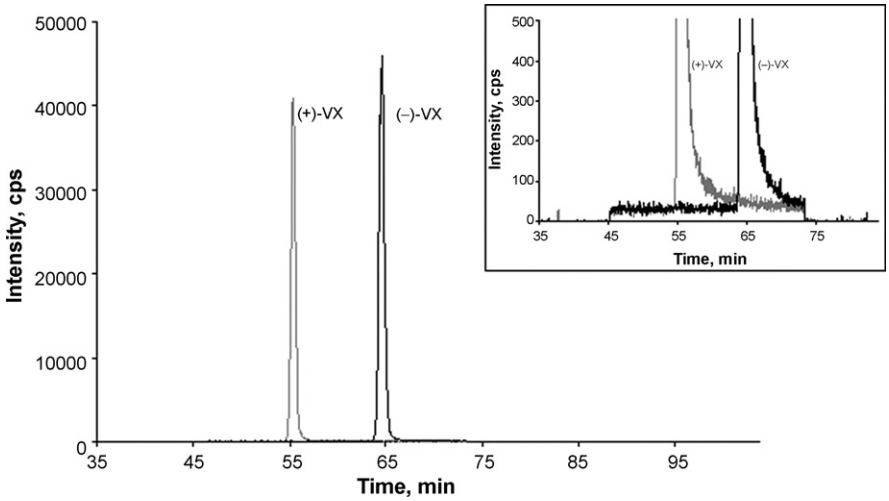

Fig. 5. Superimposed chromatograms of two fractions showing the separation of VX enantiomers on a CHIRALCEL OD-H column. LC–MS/MS analysis was performed with a CHIRAL AGP column. The inset demonstrates complete enantioselective resolution.

Table 1
The inhibition rate constant (k_i, M^{-1} min^{-1}) of racemic VX and the individual VX isomers for human AChE and BChE and swine AChE ($n = 5$)

	(−)-VX	(+)-VX	VX, racemate	$k_{i,(-)\text{-VX}}/k_{i,(+)\text{-VX}}$
AChE, human	$(1.74 \pm 0.09) \times 10^8$	$(4.57 \pm 0.12) \times 10^5$	$(9.26 \pm 0.06) \times 10^7$	381
BChE, human	$(5.69 \pm 0.57) \times 10^7$	$(2.36 \pm 0.18) \times 10^7$	$(4.18 \pm 0.11) \times 10^7$	2.4
AChE, swine	$(7.92 \pm 0.26) \times 10^7$	$(2.25 \pm 0.13) \times 10^5$	$(4.41 \pm 0.13) \times 10^7$	352

aqueous solutions of the VX isomers a slow decrease of the VX concentration was detected due to non-stereospecific hydrolysis of VX.

No differences in the response of VX (5–20,000 pg/ml) were determined in aqueous, spiked blood samples or spiked blood samples diluted with water (1:10). Consequently, samples with high VX concentrations can be diluted with water after processing without affecting quantification. Ethanol, 2-propanol and acetone resulted in an ion suppression, e.g., a lower response of VX was observed when the mobile phase A1 + B1 (Section 2.7) was modified to 5% 2-propanol in 20% methanol. In addition, 2-propanol resulted in a marked decline of the resolution of the VX enantiomers in some samples.

3.5. Recoveries

All recoveries of VX enantiomers were separately evaluated. A summary of the results is provided in Table 3.

3.6. Linearity and sensitivity

All calibration curves for the analysis of VX isomers in hemolysed blood samples $A_{(-)\text{-VX}}/A_{(+)\text{-IS}}$ versus $C_{(-)\text{-VX}}/C_{(+)\text{-IS}}$ and $A_{(+)\text{-VX}}/A_{(+)\text{-IS}}$ versus $C_{(+)\text{-VX}}/C_{(+)\text{-IS}}$ were linear within a VX concentration range of 0.5–2000 pg/ml. Representative calibration equations were $A_{(-)\text{-VX}}/A_{(+)\text{-IS}} = 0.8551 \times C_{(-)\text{-VX}}/C_{(+)\text{-IS}} + 0.0225$ ($R^2 = 1.000$) and $A_{(+)\text{-VX}}/A_{(+)\text{-IS}} = 0.9452 \times C_{(+)\text{-VX}}/C_{(+)\text{-IS}} + 0.0564$ ($R^2 = 0.999$). The limit of detection of VX amounted to approximately 200 fg per enantiomer on column. The lowest concentration of the calibration graph for (−)-VX and (+)-VX converted into mass was 750 pg, which therefore corresponded to the lower limit of quantification.

Table 2
Literature data on the ratio of the inhibition rate constants of individual VX isomers for AChE and BChE

Enzyme	$k_{i,(-)\text{-VX}}/k_{i,(+)\text{-VX}}$	Year	References
AChE, bovine erythrocytes	16.0	1977	[7]
AChE, bovine erythrocytes	146	1986	[18]
AChE, bovine erythrocytes	200	1988	[2]
AChE, human recombinant	115	2004	[15]
BChE, human (theoretical estimation)	4.4	2005	[19]

3.7. Accuracy and precision

A summary of the results on precision and accuracy as derived from the measured concentrations of VX in the spiked blood samples is shown in Table 4.

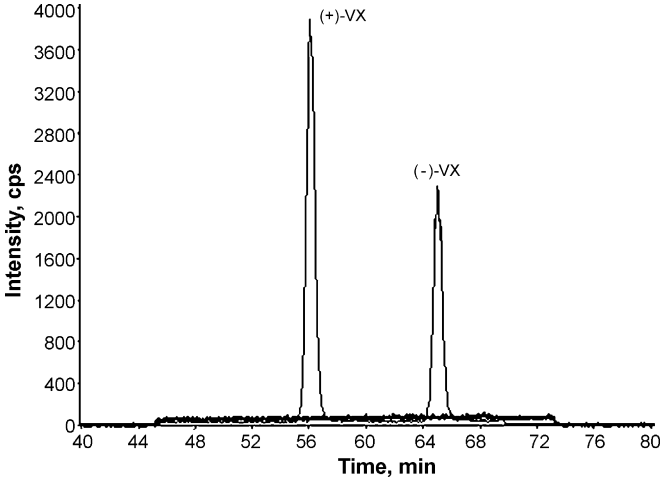

Fig. 6. LC-chromatograms of VX from an *in vivo* blood sample (thin line) and a blank blood pooled sample (thick line) (the same ion transition 268.4 → 128.2, cf. Section 2.7).

Table 3
Percentage absolute recoveries of VX in spiked hemolysed swine blood samples compared to the Internal Standard (IS) ($n = 5$)

C_{VX} (pg/ml)	Comparison to VX in water		Comparison to VX in blood matrix spiked after sample processing		Comparison to IS	
	(−)-VX	(+)-VX	(−)-VX	(+)-VX	(−)-VX	(+)-VX
8	74.4 ± 6.2	72.3 ± 8.2	72.9 ± 8.6	73.0 ± 9.3	97.2 ± 3.6	98.0 ± 3.3
80	78.1 ± 5.5	75.1 ± 8.7	72.2 ± 8.7	72.3 ± 7.0	95.2 ± 3.7	97.1 ± 4.0
400	77.3 ± 6.5	75.3 ± 4.5	74.4 ± 8.3	74.8 ± 5.2	97.3 ± 3.9	100.0 ± 4.1
2000	72.6 ± 6.8	76.3 ± 4.8	77.8 ± 3.9	76.3 ± 6.1	96.8 ± 3.9	93.7 ± 5.0

Table 4
Summary of the precision and accuracy of the analytical method for VX enantiomers in the hemolysed blood samples of swine ($n = 8$)

Racemic VX added (pg/ml)	$C_{(-)\text{-VX}}$ measured (ng/ml)	Relative standard deviation (%)	Accuracy (%)	$C_{(+)\text{-VX}}$ measured (ng/ml)	Relative standard deviation (%)	Accuracy (%)
0.0	0.0			0.0		
2	1.05	5.8	95	1.10	5.0	90
20	10.4	5.8	96	10.3	6.2	97
200	104	4.0	96	104	3.2	96
2000	970	4.6	97	1030	2.8	97

3.8. Determination of (−)-VX and (+)-VX concentration in swine blood after an i.v. and p.c. administration of racemic VX

The analytical procedure for the quantitative determination of VX isomers was applied to hemolysed blood samples from swine poisoned by intravenous and percutaneous VX. Fig. 7 shows an example of (−)-VX and (+)-VX concentrations measured in hemolysed blood samples from two swine poisoned with racemic VX and treated with i.m. atropine. The detailed toxicokinetic data will be published elsewhere. The main differences between the stereospecific toxicokinetics of VX compared to the nerve agents sarin and soman are:

- long persistence of both VX isomers, in particular after percutaneous administration,

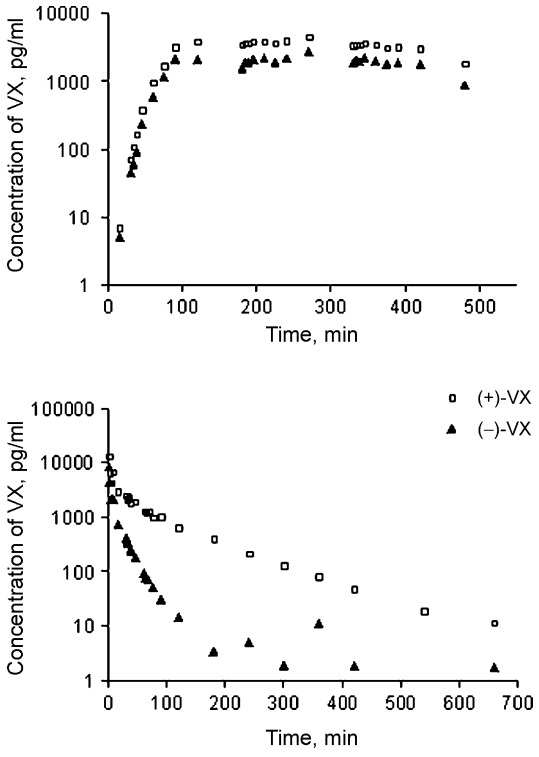

Fig. 7. Concentration of VX enantiomers in blood from a swine poisoned by a p.c. administration of 3× LD_{50} of VX (top) or by an i.v. injection of 2× LD_{50} of VX (bottom).

- higher concentration of the less toxic (+)-VX (compared to (−)-VX), especially after an intravenous injection. In case of sarin and soman injection the less toxic (+)-P isomers are either not detectable (sarin) or only transiently detectable (soman) [20–25].

Organophosphorus nerve agents bind irreversibly *in vivo* to AChE (direct toxic effect), BChE and carboxylesterase (detoxification). In addition, they are hydrolyzed by enzymes, such as paraoxonase as well as by relatively slow chemical reactions. VX is distinct in that it is hydrolytically more stable than sarin or soman. VX hydrolysis in aqueous media proceeds substantially slower than for other nerve agents and paraoxonase does not appear to inactivate VX. The unselective binding of VX to CaE is not relevant in humans or other species with low levels of this enzyme. These factors contribute to the unusually high *in vivo* selectivity of VX for AChE and results in a higher acute toxicity than other nerve agents [26–31]. The elimination of the toxic (−)-VX isomer is probably due to the specific binding to AChE.

4. Conclusions

The enantiomers of the nerve agent VX were separated by means of GC and LC (LC–MS/MS for quantification of VX enantiomers and semi-preparative HPLC with polarimetric detection for identification of VX isomers). The isolated VX enantiomers were characterised by specific optical rotation and kinetic analysis of binding to acetyl- and butyrylcholinesterase. With the developed LC–MS/MS method the enantiomeric purity of the isomers could be established. The combined method of sample preparation on basis of extraction/reconstitution and of LC–MS/MS analysis allowed baseline chiral separation of the VX enantiomers and specific and sensitive quantification of (−)-VX and (+)-VX in hemolysed swine blood. This method is characterised by high reproducibility, high selectivity and sensitivity. The application of this method was demonstrated by quantifying VX enantiomers in blood samples obtained from VX poisoned swine.

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