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Simultaneous quantification of VX and its toxic metabolite in blood and plasma samples and its application for *in vivo* and *in vitro* toxicological studies

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ABSTRACT

The present study was initiated to develop a sensitive and highly selective method for the simultaneous quantification of the nerve agent VX (O-ethyl S-[2(diisopropylamino)ethyl] methylphosphonothioate) and its toxic metabolite (EA-2192) in blood and plasma samples *in vivo* and *in vitro*. For the quantitative detection of VX and EA-2192 the resolution was realized on a HYPERCARB HPLC phase. A specific procedure was developed to isolate both toxic analytes from blood and plasma samples. The limit of detection was 0.1 pg/ml and the absolute recovery of the overall sample preparation procedure was 74% for VX and 69% for EA-2192. After intravenous and percutaneous administration of a supralethal doses of VX in anaesthetised swine both VX and EA-2192 could be quantified over 540 min following exposure. This study is the first to verify the *in vivo* formation of the toxic metabolite EA-2192 after poisoning with the nerve agent VX. Further toxicokinetic and therapeutic studies are required in order to determine the impact of EA-2192 on the treatment of acute VX poisoning.

1. Introduction

The organophosphorus compound (OP) VX (*O*-ethyl *S*-[2(diisopropylamino)ethyl] methylphosphonothioate) belongs to the most toxic group of chemical warfare nerve agents [1]. Compared to G-agents (sarin, soman and tabun) the VX enantiomers are extremely persistent in the body which presents a significant challenge in the medical treatment of acute VX intoxication [2–4]. In addition, the metabolism of VX has not been characterised in mammals and therefore the effects of possible metabolites remains to be established. In published analytical and biochemical toxicological studies only two OP metabolites of VX, EMPA (*O*-ethyl methylphosphonic acid) and MPA (methylphosphonic acid) [5–10] and two other non-phosphorus products of VX metabolism, 2-(diisopropylamino)ethanethiol DAET (formed in rat plasma under *in vitro* conditions [11]) and the methylation product of DAET 2-(diisopropylaminoethyl) methylsulphide DAEMS (identified in human serum collected from a victim of the Osaka VX incident [12]) have been evaluated. It is assumed that a third OP product of VX hydrolysis *S*-[2(diisopropylamino)ethyl] methylphosphonothioic acid (EA-2192) may be formed under *in vivo* conditions (Fig. 1). Hydrolytic degradation of VX at neutral to alkali pH (pH 7–10) results in formation of extremely stable EA-2192 [13,14]. EA-2192 (achiral analyte) was detected in VX treated silica sand, concrete, on inorganic and polymeric reactive sorbents and in decontamination solutions and environmental samples [15–20]. EA-2192 is extremely toxic, as the intravenous LD_{50} of EA-2192 in rats is 18.3 μg/kg, compared to 10.8 μg/kg for VX [21,22]. Furthermore, acetylcholinesterase (AChE) inhibited by EA-2192 and its analogues forms an instantly "aged" enzyme which cannot be reactivated by nucleophiles (oximes) [21,23,24] while AChE inhibited by VX can be reactivated successfully with oximes [25,26]. Due to the long biological persistence of VX, formation of EA-2192 in the body would substantially complicate the therapy of acute poisoning. To our knowledge, formation of the highly toxic and stable VX metabolite EA-2192 *in vivo* has not been reported. One reason for limited *in vivo* results may be the difficulty of isolating and quantifying EA-2192 at relevant *in vivo* concentrations by means of LC–MS/MS and GC–MS. EA-2192 forms non-volatile zwitterions in neutral aqueous solutions, whereupon one of the ionogenic groups is charged at all pH values except the isoelectrical point at pH 5 [21]. In addition, EA-2192 cannot be extracted with organic solvents from aqueous matrices which complicates sample preparation [16]. A number of chromatographic methods for the quantitative analysis of EA-2192 in water and diluted decontamination media were published [27–30]. However, these assays have not been designed for analysis in biological matrices, in particular for the quantification of trace concentrations (≈1 pg/ml). The aim of the present study was to develop a sensitive LC–MS/MS procedure for simultaneous and

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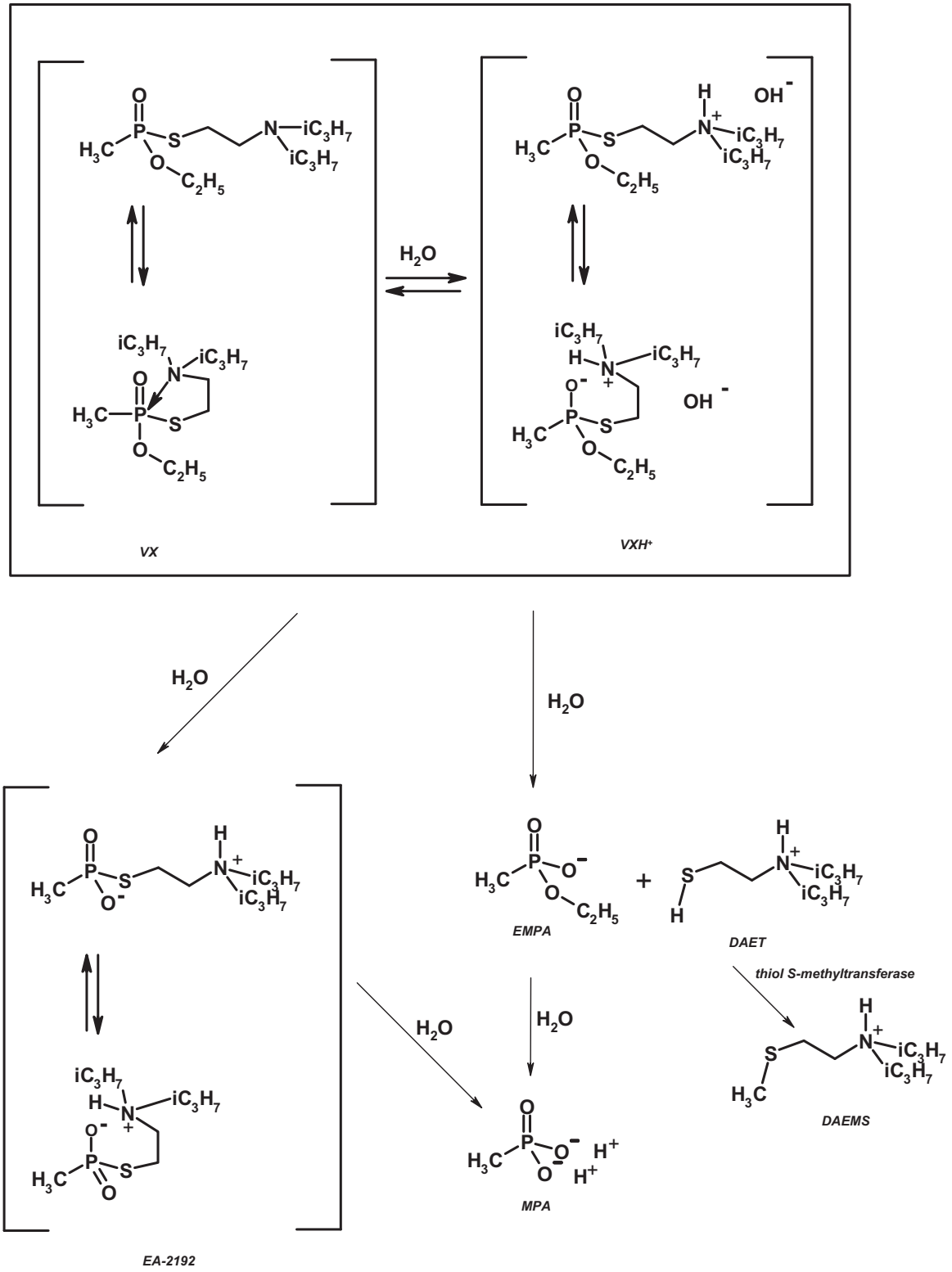

Fig. 1. Metabolism of VX.

quantitative determination of VX and EA-2192 in biological matrices at relevant concentrations for toxicokinetic and toxicodynamic studies. The procedure was applied to the quantification of VX and EA-2192 in plasma samples from *in vitro* toxicokinetic studies and 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 and HPLC columns were obtained commercially:

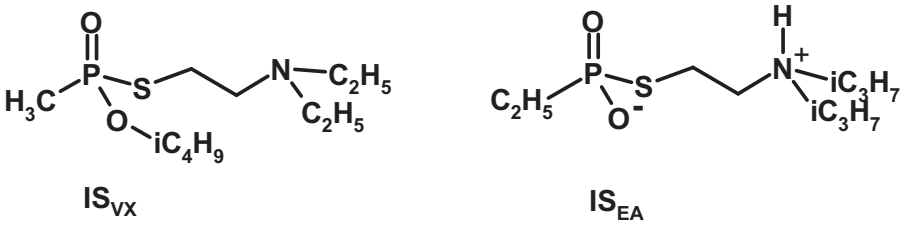

Fig. 2. Structure of internal standards.

- HYPERCARB (150 mm length, 2.1 mm i.d., 5 μm particle size) with javelin direct-connection column filter (2.1 mm i.d., one piece filter protects HPLC systems) from Thermo Fisher Scientific (Dreieich, Germany);
- deionised water (Tracepur, for organic trace analysis), n-hexane (UniSolv, for organic trace analysis), 2-propanol and methanol (SupraSolv, for gas chromatography), dimethyl sulphoxide (DMSO, SeccoSolv, dried), ammonium sulphate (Suprapur), formic acid (98–100%, analar), aqueous ammonia (25%, aristar) and obidoxime dichloride (obidoxime) from Merck (Darmstadt, Germany);
- hydrochloric acid (ISO) from Carl Roth (Karlsruhe, 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), tris(hydroxymethyl)aminomethane (Tris, Trisma Base), 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).

VX and the internal standard O-isobutyl S-[2(diethylamino)ethyl] methylphosphonothioate (IS_{VX}, >98% by GC–MS, ^{1}H NMR and ^{31}P NMR); EA-2192 and the internal standard *S*-[2(diisopropylamino)ethyl] ethylphosphonothioic acid (IS_{EA}, >95% by ^{1}H NMR and ^{31}P NMR) (Fig. 2) were made available by the German Ministry of Defence, Bonn. VX of the same quality for *in vivo* experiments was synthesized at the Canadian National Single Small Scale Facility at DRDC Suffield.

HI-6 dichloride (HI-6) was a gift from Dr. Clement (Defence Research and Development Canada – Suffield, Alberta, Canada).

Heparinised human blood was supplied by the Bavarian Red Cross Blood Bank (Augsburg, Germany). Heparinised human and swine blood samples were centrifuged (3000 min^{-1}, 4 °C, 15 min), plasma separated from the erythrocytes and pooled. Heparinised pooled plasma from Wistar rats, NMRI mice, New Zealand white rabbits and Dunkin-Hartley guinea pigs was obtained from Charles River (Sulzfeld, Germany). Hemoglobin-free human and swine erythrocyte ghosts were prepared as described before [31] and served as source for human and swine acetylcholinesterase (AChE, E.C. 3.1.1.7).

2.2. Standards and spiking solutions

Standard solutions of VX and IS_{VX} (1%, w/v in hexane), EA-2192 and IS_{EA} (1%, w/v in DMSO) were prepared for analytical experiments and were used instantly for preparation of stock solutions in 2-propanol (1 μg/ml for each analyte). The stock solutions were stored for up to one month at a room temperature in glass vials. The stock solutions were further diluted in deionised water (spiking solution) on the day of the experiment. In blood and plasma samples, the volume of spiking solution did not exceed 1% (v/v) and the final concentration of 2-propanol was less than 1%.

EA-2192 stock solutions for enzyme kinetic experiments (1% in DMSO) were prepared weekly, stored at room temperature and appropriately diluted in distilled water just before the experiment. Obidoxime and HI-6 (200 mM) were prepared in distilled water, stored at −80 °C and diluted as required in distilled water on the day of the experiment. All daily prepared solutions were kept on ice until used in the experiment.

2.3. Instrumentation

The analytical LC–MS/MS system for the quantification of VX and EA-2192 consisted of two binary LC-20AB pumps (pump 1 and pump 2, Fig. 3), 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).

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 (Varian, Darmstadt, Germany).

The degradation of VX in plasma of different species was investigated by using a Titrando 836 titration system for incubation and sampling (Metrohm, Filderstadt, Germany).

2.4. Laboratory animals, in vivo experiments and blood sampling

2.4.1. Domestic swine experiments

Castrated male York-Landrace cross swine (~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 one week prior to experimental use. Animals were fed until the evening prior to experimentation 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 all monitors and catheters were placed and affixed, isoflurane was

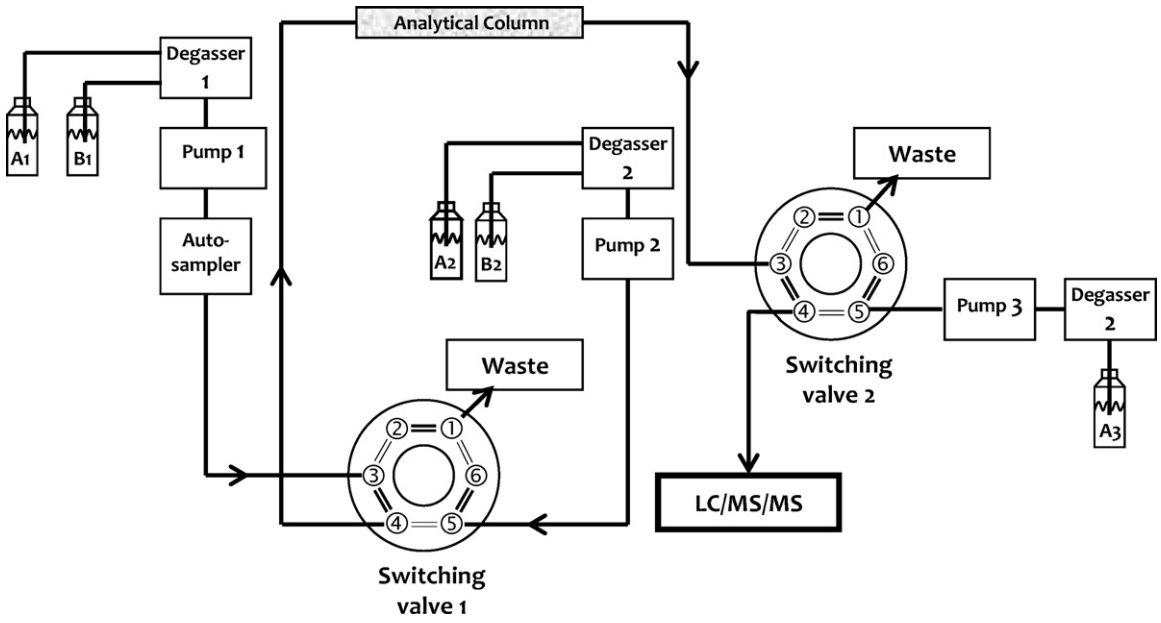

Fig. 3. Schematic diagram of LC–MS/MS with switching valves, javelin direct-connection column filter is not showed.

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 with a Hallowell EMC Model 3000 ventilator (Pittsfield, MA, USA) at a rate of 150–200 ml per cycle at 20 cycles per minute.

2.4.3. Nerve agent administration

A 2× LD_{50} dose of VX, 24.6 μg/kg [2], 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%. For percutaneous, a 3× LD_{50} dose of neat VX, 186.9 μg/kg [2], was pipetted onto a clipped area on the inner surface of the ear pinna.

2.4.4. Medical countermeasures

Atropine sulphate (AS; 0.03 mg/kg) was injected i.m. at 1, 31, 61 min and at 30, 180, 330 min post-exposure in i.v. and p.c. poisoned animals, respectively. Assisted ventilation was provided as required at a rate 3–4 l min^{-1}. Control animals (n = 6, each) received AS i.m. at 1 min (i.v.) or 30 min (p.c.) post-exposure to vehicle.

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, 8, 16, 31, 32, 34, 38, 46, 61, 62, 64, 68, 76, 90, 120, 180, 240, 300, 360, 420 min (for i.v. application of VX) and 0, 5, 15, 30, 34, 45, 60, 75, 80, 85, 90, 120, 184, 188, 210, 225, 240, 270, 330, 334, 338, 345, 360, 375, 390, 420, 480, 540 min (for p.c. application 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 6 swine for i.v. and 6 swine for p.c. studies were investigated. For experiments with blank and spiked samples individual and pooled hemolysed blood from 12 healthy swine were used. Individual hemolysed blood samples were used for the study of specificity. The sample processing for hemolysed blood samples and for *in vivo* samples was identical.

2.5. Sample preparation

Stabilization of VX and EA-2192 was achieved by successive treatment of the *in vivo* hemolysed blood samples with two formate buffers as described before [2]: 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.

The sample processing procedure was optimized for the most selective removal of endogenous contaminants in order to minimize disturbance of chromatographic resolution of VX, EA-2192, IS_{VX} and IS_{EA} and quantification of VX and EA-2192. This procedure included a combination of protein precipitation, liquid extraction and re-extraction/reconstitution. The samples were thawed quickly under flowing cold water and kept in an ice bath until further processing (less than 15 min). Four milliliters of each sample were used for further treatment, while the remainder was frozen again to serve as reference sample for an optional repetitive analysis. Immediately prior to sample preparation the samples were spiked with IS_{VX} and IS_{EA} at a final concentration of 100 pg/ml of each. Protein precipitation and extraction were accomplished according to the following scheme. Eight grams of ammonium sulphate were added to each sample. The samples were gently mixed 30 s followed by protein precipitation/extraction with 32 ml of an organic mixture (2-propanol, hexane, methanol 1:1:1, 0 °C). After centrifugation (4000 × g, 4 °C, 15 min) the supernatant (two liquid phases) was transferred quantitatively to a Turbo Vap LV work station. The aqueous organic phases were evaporated at 40 °C with nitrogen (25 psi) up to 1 ml. 10 ml of 2-propanol were added to each sample and the evaporation was carried out up to 1 ml again. Addition of 2-propanol and evaporation was repeated two times. Subsequently, 2 ml of tetradecane were added to each sample and the evaporation was continued up to “dry” tetradecane (without aqueous phase on the bottom of the glass tube). Then, the reconstitution was carried out by adding 1.5 ml deionised water. After centrifugation (8200 × g, 40 min at 0 °C) of each sample a defined

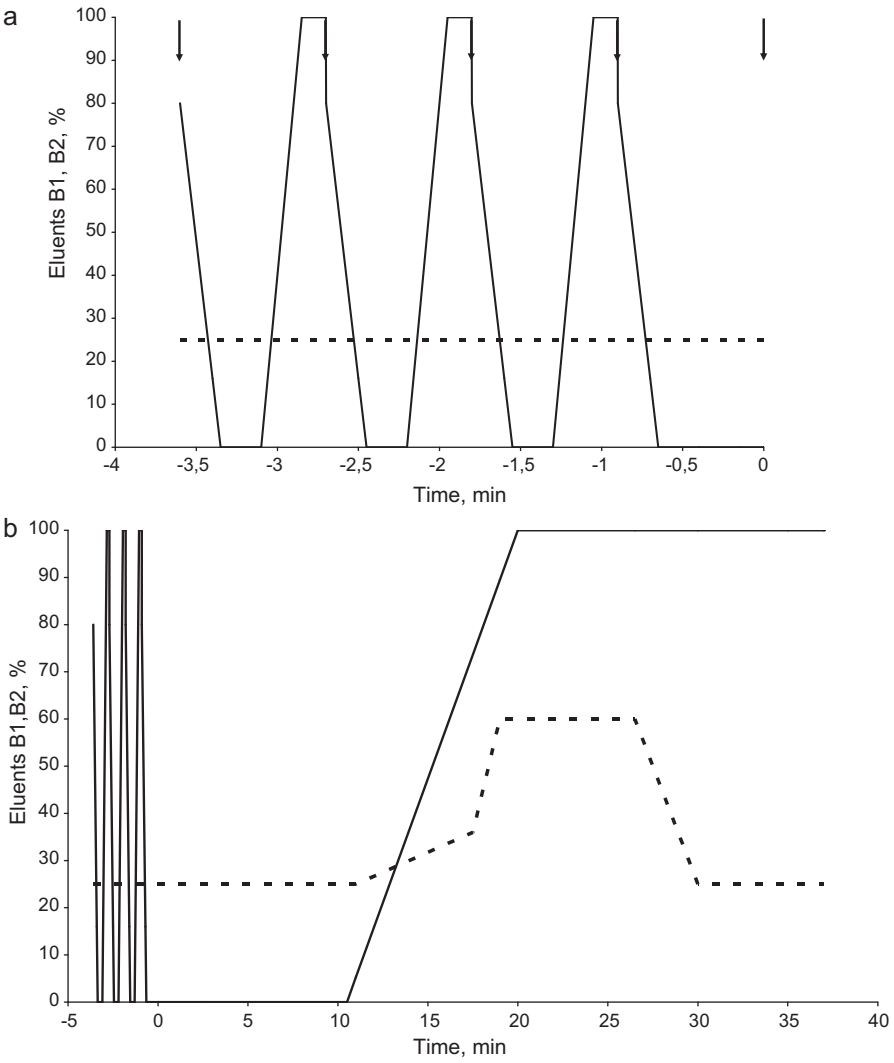

Fig. 4. Flow diagram of the eluents: eluent B1 (solid line), eluent B2 (dashed line), arrows mark injections (figure *a*, 5 × 100 μl). Mobile phases A1 and B1 were pumped through the column into the waste between −3.25 and 7 min (injections-trap-rinse). Mobile phases A2 and B2 were pumped through the column between 7 and 32 min (into mass spectrometer between 10 and 32 min, figure *b*).

volume of the intermediate phase (1.2–1.4 ml) was diluted 1:1 with deionised water and transferred to an autosampler vial for LC–MS/MS analysis.

2.6. LC–MS/MS analysis

The mobile phases consisted of deionised water (eluent A1, Fig. 3), 25 mM ammonium formate buffer pH 9.0 (eluent B1), 25 mM ammonium formate buffer pH 5.0 (eluent A2), methanol (eluent B2), deionised water–methanol (9:1, v/v, eluent A3). Large volume injection technique (LVI) with pulse eluents gradient (ammonium formate buffer pH 9.0 – deionised water, Fig. 4) was used. Each sample (5 × 100 μl) was injected and analytes were concentrated on the initial sector of an analytical HYPERCARB column, followed by a 7 min rinse with deionised water (eluent A1). The total flow rate of the eluents A1–B1 and A2–B2 through the column was kept at 140 μl min^{-1}. The column was thermostatted at 30 °C. The LC resolution was achieved with the application of the following linear gradients as illustrated in Fig. 4. The rinse of the analytical column between 7 and 11 min (after the switching of the six port valve 1) was continued with eluents A2 and B2 (25%). After the rinse of the analytical column eluent B2 was increased from 25% to 36% within 6.5 min and to 60% within 2.5 min, this condition was maintained for the next 7.5 min, then the system was re-equilibrated to 25% eluent B2 within 3.5 min and maintained for 7 min.

The instrument parameter for positive electrospray ionisation were as follows: nitrogen as curtain gas (11 psi), collision gas (setting medium), ion source gas 1 (10 psi) and ion source gas 2 (10 psi), the interface temperature was 300 °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.

The precursor $[M+H]^+ \rightarrow$ dialkyl aziridinium cations (products of the cleavage of the S–C bonds [32]) transitions ion masses used for multiple reaction monitoring (MRM) mode were: m/z 268.4 → 128.2 (VX), m/z 268.4 → 100.2 (IS_{VX}), m/z 240.3 → 128.2 (EA-2192), m/z 254.3 → 128.2 (IS_{EA}), dwell-time was 0.6 s per transition.

2.7. Determination of inhibition rate constants of EA-2192 and reactivation of EA-2129 inhibited AChE by oximes

Acetylcholinesterase (AChE, E.C. 3.1.1.7) and BChE activities were measured spectrophotometrically at 412 nm using a modified Ellman assay [33]. 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.

EA-2192 second order inhibition rate constants (k_i) were determined as described before [25]. In brief, 10 μl human or swine erythrocyte ghosts and 5 μl diluted EA-2129 (0.75–2.25 μM final concentration) were added to a cuvette containing phosphate buffer, DTNB and ATCh (final volume 3.165 ml). ATCh hydrolysis was continuously monitored for up to 30 min. The recorded curves were analyzed by non-linear regression analysis and used for the further determination of k_i. The low inhibitory potency of EA-2129 with human BChE required the determination of the inhibition rate constant k_i with a different approach [26]. In brief, a small volume (1%, v/v) of appropriately diluted EA-2129 (5–20 μM final concentration) was added to temperature-equilibrated (37 °C) BChE at $t = 0$. Aliquots were removed after specified time intervals (1–9 min) and transferred to a cuvette for the determination of residual enzyme activity which was referred to control activity.

Human and swine erythrocyte ghosts were incubated with EA-2192 in order to prepare inhibited AChE for reactivation studies. EA-2192 (500 nM final concentration) was added to ghost preparations for 15 min at 37 °C to achieve 95–98% inhibited AChE. Then, treated ghost were dialyzed (phosphate buffer, 0.1 M, pH 7.4) overnight at 4 °C to remove residual inhibitor [34]. The ability of obidoxime and HI-6 to reactivate EA-2129 inhibited human and swine AChE was tested with different experimental protocols. First, inhibited AChE was incubated with obidoxime or HI-6 (1 mM) and aliquots were taken to determine the enzyme activity after 0 – 30 min. In a second set of experiments, EA-2129 treated human and swine erythrocyte ghosts were mixed with soman-treated human plasma [34] in order to stabilize AChE activity during long-term incubation at 37 °C. Then, obidoxime or HI-6 (1 mM) was added and aliquots were taken to determine the enzyme activity after 0–24 h. Finally, EA-2129 inhibited human and swine AChE was highly diluted (300fold), HI-6 (20 μM) was added and the enzyme activity was determined after 10 min. AChE activities were referred to adequately treated controls and are given as % reactivation.

The analysis of the data was performed with Prism™ Version 4.00 (GraphPad Software, San Diego, CA, USA).

2.8. Study of specificity

The specificity of the LC–MS/MS method was checked by analysing six individual hemolysed blood samples spiked with VX, EA-2192 and internal standards and six individual blank hemolysed blood samples. All chromatograms were compared with chromatograms from VX standard solutions. *In vivo* blood, spiked pooled plasma samples as well as diluted plasma samples after the *in vitro* toxicokinetic studies were also used for additional comparison.

2.9. Determination of recovery rates

The absolute recovery rates were calculated by comparing the chromatogram peak areas of VX and EA-2192 in spiked swine pooled hemolysed blood (spiked prior to sample preparation) 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 three concentrations (2.5, 25, 250 pg/ml; $n = 5$ at each concentration). The recovery compared to IS_{VX} (IS_{EA}) was determined by comparing peak area ratios obtained from analytes to IS_{VX} (IS_{EA}) versus the peak area ratios of the same concentration of VX (EA-2192) standards and IS_{VX} (IS_{EA}) spiked in treated hemolysed blood samples. Identical concentrations of analytes were used for the determination of the absolute recovery.

2.10. Study of linearity and sensitivity

Linearity test calibration curves were generated within a VX (EA-2192) concentration ranges of 0.5–300 pg/ml by using spiked swine pooled hemolysed blood samples ($n = 5$). The linearity of each pair calibration curves was determined by plotting the peak-area ratios A_{VX}/A_{ISVX} versus C_{VX}/C_{ISVX} and A_{EA}/A_{ISEA} versus C_{EA}/C_{ISEA} 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.

2.11. Accuracy and precision

Accuracy and precision of the analytical procedure was tested at three VX (EA-2192) concentrations (2.5, 25, 250 pg/ml; $n = 8$, each) in swine pooled hemolysed blood samples.

2.12. VX and EA-2192 quantification in in vivo and in vitro blood and plasma samples

The applicability of the analytical LC–MS/MS assay was tested in samples from *in vitro* and *in vivo* toxicokinetic studies. For the evaluation of calibration curves pooled blood swine samples or plasma samples from different species were spiked with VX and EA-2192 (0.5–300 pg/ml) and the internal standards IS_{VX} and IS_{EA} (100 pg/ml). Calibration curves were generated for each analytical run. To investigate the degradation of VX in plasma samples heparinised human, swine, guinea pig, rabbit, rat and mice plasma was diluted 1:5 with Tris–HCl buffer pH 7.40 and was incubated with 1 μM VX for up to 6 h at 37 °C. The incubation details and the sampling were carried out as described before [35] with slight modification. 200 μl diluted plasma was mixed with two sodium formate buffers [2] followed by the sample preparation described in Section 2.5. For exclusion of possible interferences the analytical procedure was partially validated, i.e. linearity, specificity, sensitivity, accuracy and precision with diluted plasma of different species (human, swine, rabbit, guinea pig, rat, mice, data not shown).

3. Results and discussion

3.1. Development of the sample preparation and HPLC method with LVI mode

The isolation and subsequent quantification of the toxic VX metabolite EA-2192 in biological matrices at trace concentrations has been limited by experimental difficulties (cf. Section 1). To overcome these difficulties and optimize the analytical procedure for the simultaneous quantification of VX and EA-2192 an evaluation should be directed on the specific physicochemical properties of these analytes. According to some publications, the pK_a-value of the VX amino group was determined to be 8–9. Hence, the nitrogen atom of VX and probably of other derivatives of *S*-[2(dialkylylamino)ethyl] methylphosphonothioic acids are protonated to a considerable extent at neutral/physiological pH. Nevertheless, up to 10% of VX with a tertiary nitrogen atom are present under these conditions [14,36–38]. The phosphorus–oxygen double bond assigns partially ionic character [39], the electrostatic interactions of the phosphorus or oxygen atoms of the phosphoryl group with the amino group (either uncharged tertiary or positive charged quaternary nitrogen atom of the protonated VX and its analogues) could result in the formation of cyclic structures in solutions (with or without participation of H_2O molecules) (Fig. 1). Interactions of charged nitrogen and oxygen atoms of zwitterions EA-2192 (electrostatic stabilisation) could generate cyclic structures as well [37,40–42]. We assumed

Table 1
Partition coefficients (P) of VX between organic solvents and water (derived from data [43,44]).

Solvent	P	T (°C)
n-Hexane	3.5	25
Octanol	4.7	Room
Toluene	6.8	25
1,2-Dichloroethane	21.4	25

that several equilibriums take place in solutions of O-alkyl *S*-[2(dalkylylamino)ethyl] methylphosphonothioates: between the protonated and unprotonated forms of the open structure and between various forms of cyclic structures. Experimentally determined partition coefficients of VX in organic solvents–water systems confirm an important role of the unprotonated form of this substance [43,44] (Table 1).

Further, we assumed that the hydrophobic interactions between the tertiary amino group of VX and EA-2192 and the molecular surface of the stationary phases are important factors for the retention effect of the analytes. The enantioresolution of VX could be realized earlier on α_1-acid glycoprotein phase exclusively in basic buffer systems (pH 9), which confirms an importance of the molecular interactions between the tertiary amino group of VX and the amino acids of the chiral selector [2].

The equilibrium between the tertiary amino group and the quaternary amino group could be shifted either in the direction towards uncharged amine by alkali buffer and concentrated salt systems (solutions or oversaturated suspensions) or in direction towards ammonium derivatives by acidic buffer systems; this was used in the present study for the extraction clean up step and the sample loading in the LVI mode. The use of salt suspensions on the basis of $MgSO_4$ for salting out in different clean up/extraction/partitioning procedures were applied earlier for the sample preparation and subsequent quantification of pesticides in vegetable materials (cf. QuEChERS method [45,46]). After preliminary experiments with different salt mixtures and extraction/protein precipitation procedures the quantitative ratios of hemolysed blood, ammonium sulphate and organic solvents were optimized. Finally, a relatively simple sample preparation method including treatment of hemolysed blood samples with excess of ammonium sulphate followed by the extraction with a cold organic solvents mixture (hexane, 2-propanol and methanol) was applied. In the successive evaporation – extraction clean up step tetradecane was added to the extracts to prevent the evaporation loss of the analytes and to allow removal of hydrophobic endogenous impurities prior to the final re-extraction – reconstitution with deionised water.

The HYPERCARB (porous graphite carbon, PGC) material as a particular stationary phase with a broad spectrum of interactions to the analytes was selected in the early development stages of the presented assay. A combination of eluents on the basis of alkaline and acidic ammonium formate buffers was used for the retention and elution of VX and EA-2192 on/from the chromatographic column. The parameters for the HPLC separation, i.e. injection volume, injection and elution profiles, composition of mobile phases, pH of buffer systems and proportion of methanol–ammonium formate buffers were optimized during method development. Different HPLC set-ups in LVI mode including trap columns (e.g. guard-columns) prior to the analytical column were tested in preliminary experiments. The best results were achieved with the "one column mode". A single sample loading with large volumes [2] revealed an unacceptable chromatographic performance because of the high load of the HYPERCARB columns with matrix and impurities. Compared to an AGP column, the HYPERCARB retains essentially more impurities over a period of injection, which prevents a focussing of the analytes on the initial sector of the column [2]. In order to solve this problem, a pulse gradient LVI was applied: *injection and trap in basic buffer – a rinse with a gradient basic buffer – deionised water plus a final singular rinse with deionised water and acidic buffer plus methanol after the switching of the HPLC pumps from 1 to 2* (Fig. 3); a performance comparison of two LVI mode "with/without basic buffer" is presented in Fig. 5. The final HPLC procedure involved five injections of 100 μl sample, although up to ten injections could be carried out without affecting the detector linearity (data not shown).

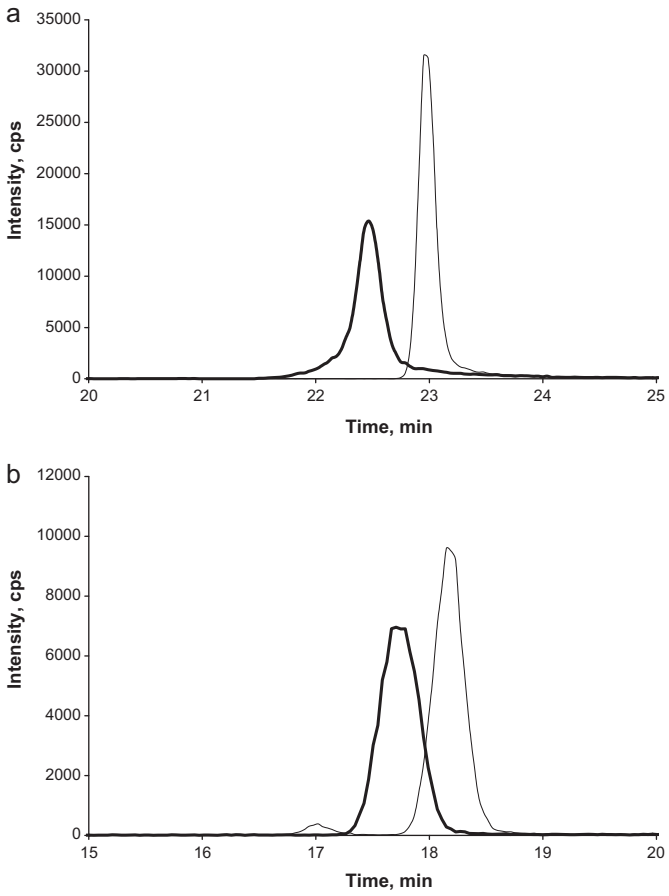

Fig. 5. Influence of the eluents in the injection mode on the peak form of VX (*a*) and EA-2192 (*b*): 5 × 100 μl were injected with eluent A1 isocratic (thick line) and with pulse eluents gradient as in Fig. 4 (thin line). Representative MRM chromatograms of VX (268.4 → 128.2, *a*) and EA-2192 (240.3 → 128.2, *b*).

3.2. Specificity of the LC–MS/MS assay

The peaks of VX and EA-2192 were selectively detectable in all standard solutions of VX and EA-2192 in water, ammonium formate buffer pH 9.0, spiked blank blood (plasma) and hemolysed *in vivo* blood samples. No interfering peaks of endogenous and sample processing impurities from blood or plasma diluted with formate buffers were found in chromatograms of blank pooled, individual, *in vivo* blood and plasma samples (Fig. 6). The peak form and the retention time of both analytes did not vary in standard, spiked or *in vivo* samples when using the same column, although small differences (up to 0.9 min) in retention times were observed with different columns.

VX and EA-2192 were shown to be stable in stabilized formate buffer solutions at −80 °C for at least 18 months. Both analytes were also stable in samples after two freeze–thaw cycles and incubation in an ice bath for at least 1 h.

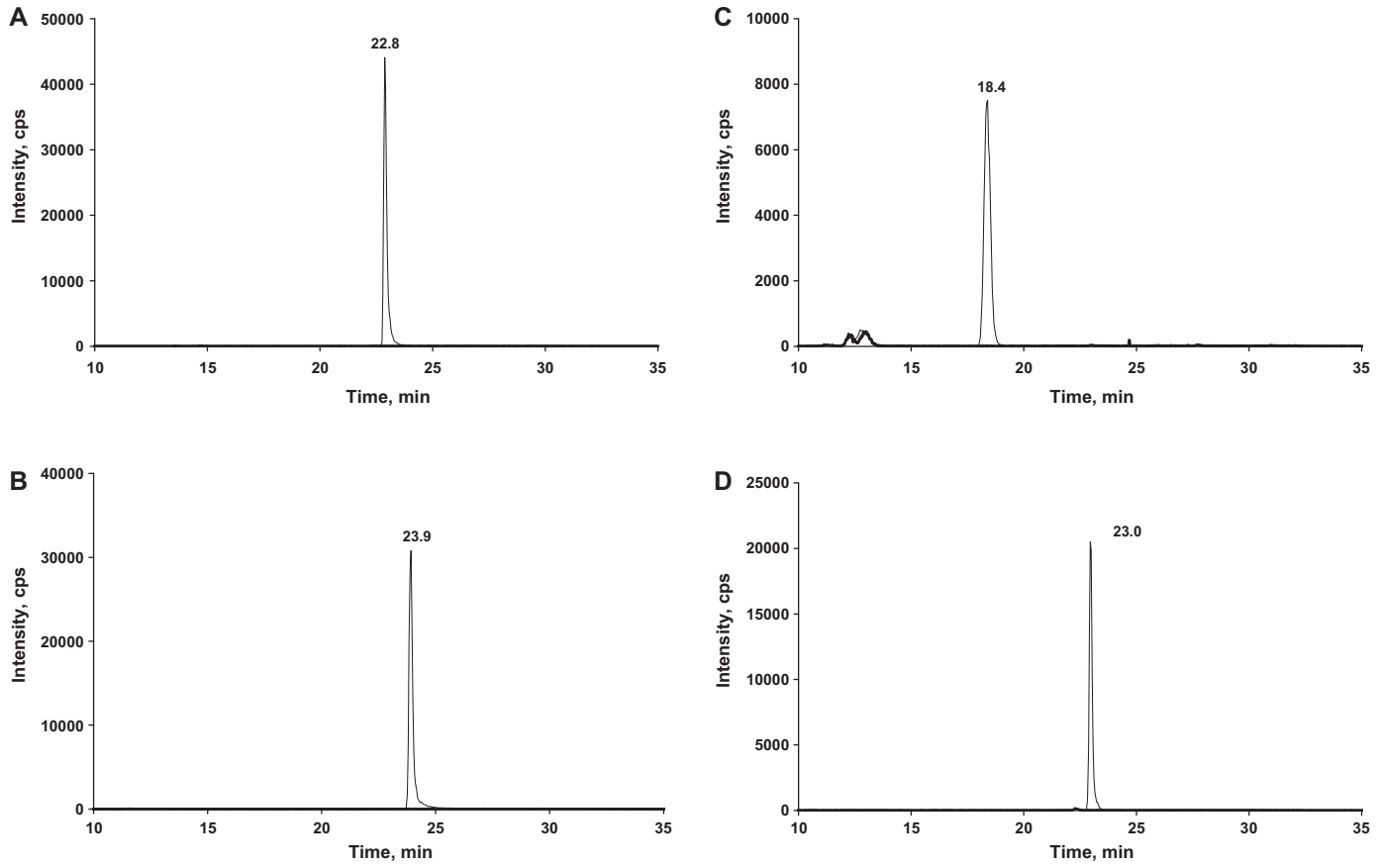

Fig. 6. Representative MRM chromatograms of VX (268.4 → 128.2, *a*), IS_{VX} (268.4 → 100.2, *b*), EA-2192 (240.3 → 128.2, *c*) and IS_{EA} (254.3 → 128.2, *d*) in *in vivo* blood sample (thin lines) and blank pooled swine blood sample (thick lines). Retention times are indicated over the peaks.

No differences in the response of VX and EA-2192 were determined in aqueous, spiked blood and plasma samples or spiked blood and plasma samples diluted with water (up to 1:100). Consequently, samples with high VX or EA-2192 concentrations could be diluted with water after processing without affecting quantification. Without the dilution step after the reconstitution with deionised water the interference of the matrix components resulted in a marked ion suppression of EA-2192 in some samples.

3.3. Recoveries

The results of the recovery study are shown in Table 2. EA-2192 recovery rate (69%, average value for three concentrations) was slightly below the recovery rate of VX (74%). Quantification data of EA-2192 in biological matrices are not available in the open literature while VX recovery rates of 65% (human plasma [47]), 75% (swine hemolysed blood [2]) and 80–90% (one step direct extraction from guinea pig blood [48]) were published.

3.4. Linearity and sensitivity of the LC–MS/MS assay

All calibration curves for the analysis of VX and its toxic metabolite in hemolysed pooled blood and plasma samples A_{VX}/A_{VXIS} versus C_{VX}/C_{VXIS} and A_{EA}/A_{EAIS} versus C_{EA}/C_{EAIS} were linear within a VX concentration range of 0.5–300 pg/ml. Representative calibration equations were $A_{VX}/A_{VXIS} = 0.7662 * C_{VX}/C_{VXIS} + 0.0141$ ($R^2 = 0.999$) and $A_{EA}/A_{EAIS} = 0.7905 * C_{EA}/C_{EAIS} + 0.0217$ ($R^2 = 0.997$). The limit of detection (LOD) of the both analytes amounted to approximately 0.1 pg/ml per substance and the lower limit of quantification (LOQ) was 0.5 pg/ml.

3.5. Accuracy and precision

A summary of the results on precision and accuracy as derived from the measured concentrations of VX and EA-2192 in spiked blood samples are shown in Table 3.

3.6. Determination of VX and EA-2192 concentrations in blood and plasma samples after in vivo and in vitro toxicokinetic studies: combined consideration with AChE/BChE inhibition and reactivation data

The developed assay for the simultaneous quantitative determination of VX and its toxic metabolite EA-2192 was applied to hemolysed blood samples from swine poisoned by intravenous and percutaneous VX as well as to VX-treated human, swine, rat, rabbit, guinea pig and mice plasma samples. Figs. 7 and 8 show representative examples of VX and EA-2192 concentration profiles measured in hemolysed blood samples from two swine poisoned with VX and treated with i.m. atropine as well as *in vitro* degradation of VX and the formation of EA-2192 in rat plasma diluted with Tris–HCl buffer. A detailed toxicokinetic analysis would be beyond the scope of this publication.

In vivo, both VX and its toxic metabolite EA-2192 persisted for a prolonged time, in particular after a percutaneous exposure to VX (Fig. 7). The kinetic profile of the both substances followed a comparable course after p.c. VX application (Fig. 7 bottom). Intravenous VX administration resulted in a parallel terminal elimination phase of VX and EA-2192 (Fig. 7 top). Probably the toxic metabolite forms in tissues and is cleared at a comparable rate as VX. Based on

Table 2
Percentage of absolute recoveries of VX and EA-2192 in spiked hemolysed pooled swine blood samples ($n = 5$).

$C_{VX/EA-2192}$ (pg/ml)	Comparison to corresponding analyte in water		Comparison to corresponding analyte in blood matrix spiked after sample processing		Comparison to corresponding IS	
	VX	EA-2192	VX	EA-2192	VX	EA-2192
2.5	70.6 ± 5.0	65.3 ± 7.4	72.2 ± 9.2	68.0 ± 9.0	94.9 ± 3.9	96.4 ± 3.6
25	72.1 ± 5.9	68.1 ± 6.3	74.7 ± 6.7	68.8 ± 7.3	96.8 ± 4.4	96.8 ± 3.6
250	75.5 ± 6.0	69.3 ± 5.5	75.1 ± 6.3	70.9 ± 5.1	97.9 ± 4.0	99.0 ± 3.2

Table 3
Summary of the precision and accuracy of the analytical assay for VX and EA-2192 in spiked hemolysed swine blood samples ($n = 8$, each, inter-assay data).

VX/EA-2192 added (pg/ml)	C_{VX} measured (pg/ml)	Relative standard deviation (%)	Accuracy (%)	$C_{EA-2192}$ measured (pg/ml)	Relative standard deviation (%)	Accuracy (%)
0.0	0.0			0.0		
2.5	2.25	5.0	90	2.30	5.0	92
25	24.0	4.7	96	27.0	5.5	93
250	235	4.4	94	264	4.2	95

published results, a number of enzyme systems could mediate deethylation reactions of VX [49].

Rat plasma exhibited the highest VX detoxification activity of investigated species, resulting in degradation half-times of 25 min (rat), 180 min (mouse), 300 min (rabbit), 900 min (guinea pig, human) and 1200 min (swine). It may be assumed that VX is degraded by enzymes of the carboxylesterase (CaE) family since rodent plasma with rather high CaE concentrations (rat, mice, rabbit) exhibited a higher degradation activity towards VX [50–52].

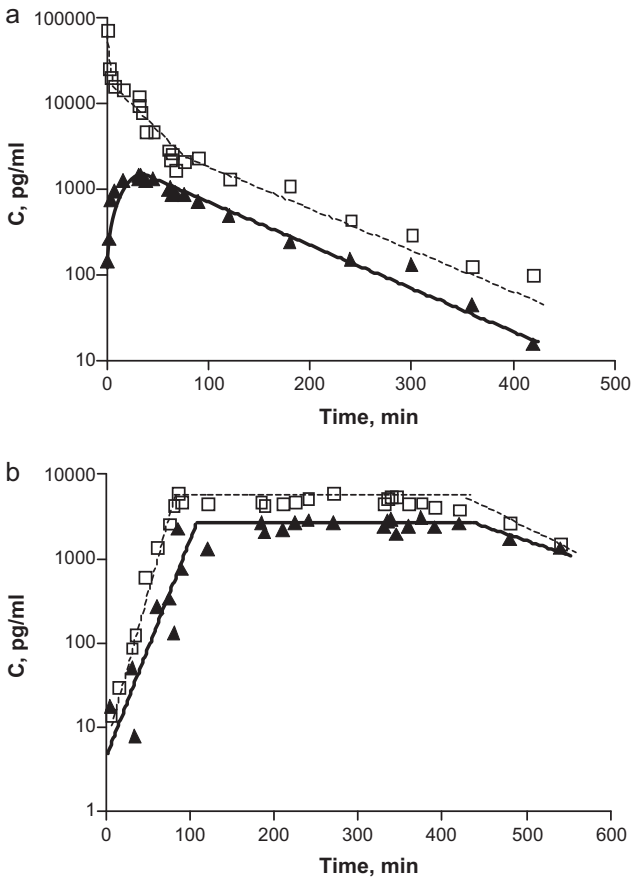

Fig. 7. Concentration of VX (squares) and EA-2192 (triangles) in whole blood from a swine poisoned by an i.v. injection of 2× LD_{50} of VX (*a*) or by a p.c. administration of 3× LD_{50} of VX (*b*). The lines were integrated to visualize the different absorption and elimination phases and were not intended to calculate toxicokinetic parameters.

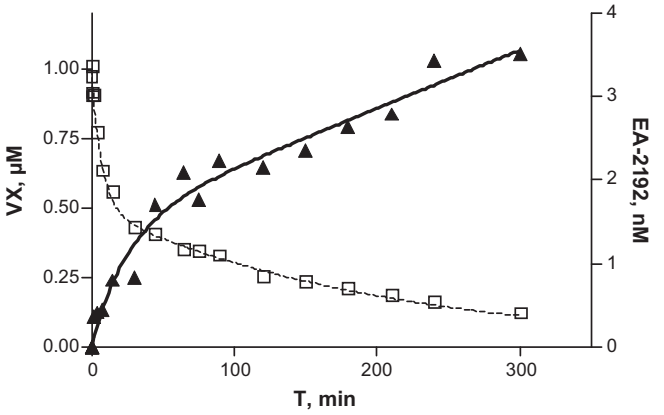

Fig. 8. Degradation of VX (dashed line and squares) and formation of EA-2192 (solid line and triangles) in heparinised pooled rat plasma diluted 1:5 with Tris–HCl buffer pH 7.40 and spiked with 1 µM VX.

The kinetics of EA-2192 formation was comparable in plasma of all tested species and in Tris–HCl buffer pH 7.4 indicating that the mechanism was not enzymatic.

Data on the inhibition kinetics of VX and EA-2192 with human and swine AChE and human BChE are presented in Table 4. Compared to VX, EA-2192 had a lower inhibitory potency with both enzymes, the difference was more pronounced with BChE. The anticipated formation of a cyclic structure of O-dealkylated methylphosphonothioates may result in a decreased affinity to the active center of cholinesterases in comparison to VX [53,54]. Despite of a remarkable lower inhibitory potency of EA-2192 with AChE and BChE this agent was found to be almost as toxic as VX in rats [21,22]. This discrepancy may be attributed in part to a low affinity of EA-2192 to BChE and probably to carboxylesterases (CaE). CaE's are inhibited mainly by hydrophobic OPs and weakly with cationic OPs [55–57] whereas EA-2192 is a zwitterionic and hydrophilic substance. In addition, the physicochemical properties

Table 4
Inhibition rate constants (k_i) of VX and EA-2192 for human and swine cholinesterases ($M^{-1}\,min^{-1}$).

	Human			Swine
	AChE	BChE	AChE/BChE	AChE
VX	9.91×10^7	4.18×10^7	2.37	4.41×10^7
EA-2192	1.51×10^6	4.75×10^3	317	5.52×10^5
VX/EA-2192	65.8	8800		79.9

of EA-2192 may result in an exclusive extracellular distribution of the compound thus increasing its toxicity in comparison to uncharged analoques [58]. This assumption was confirmed by rat studies with supralethal EA-2192 doses showing an almost exclusive peripheral action of the compound [21].

Incubation of EA-2129 inhibited human and swine AChE with obidoxime and HI-6 did not result in any increase of AChE activity. These findings confirm previous results on the inability of the oximes TMB-4 and P2S to reactivate AChE inhibited by EA-2192 and the n-propyl analogue [21,23]. Phosphonylation of the active site serine by EA-2192 results in the formation of an immediately aged AChE.

In conclusion, a sensitive and selective LC–MS/MS procedure for the quantification of EA-2192 in biological samples from different species was developed and applied to samples from *in vivo* and *in vitro* toxicokinetic studies. EA-2192 was shown to be notably stable in biological systems, to be a potent selective AChE inhibitor and to form a phosphonylated AChE which is resistant towards reactivation by oximes. Further toxicokinetic and therapeutic studies are required in order to determine the impact of EA-2192 on the treatment of acute poisoning by VX.

Conflicts of interest statement

The authors declare that they do not have any conflicts of interest.

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