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Methodological contributions towards LC–MS/MS quantification of free VX in plasma: An innovative approach

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ABSTRACT

A novel analytical method has been developed to detect and quantify VX (O-ethyl *S*-(2(diisopropylamino) ethyl) (methylphosphonothioate)) in plasma using an LC–MS/MS technique. VX detection and quantification in plasma following percutaneous exposure represent a formidable challenge and it is an important part of the ongoing struggle against chemical warfare agents. Liquid–liquid extraction of VX from plasma was performed and it generated a recovery rate of approximately 65% followed by an LC–MS/MS analysis in a 100% organic phase. An Allure biphenyl column (Restek) was tested with detection limit at 0.5 pg/mL (5 μL injected). Initial application was focused on human skin grafted on nude mice as an experimental model with proper adjustments done for very small quantities of plasma.

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1. Introduction

Organophosphorus (OP) nerve agents are among some of the most studied chemical warfare agents especially the V series due to their acute toxicity and the potential threat they pose to the general public at large [1]. In the V series, VX also known as O-ethyl *S*-[2(diisopropylamino) ethyl] methylphosphonothioate is extensively studied in toxicology. Medical investigations already conducted in the field of toxicology to determine VX toxicity encompass both curative [2–5] and preventive measures [6–8]. Quantitative measurement of blood cholinesterase activity and OP metabolites (phosphonic acids) in biological fluids provides relevant indicators about the toxicity [9–12] level. In addition to medical research, prophylactic or protective measures such as decontamination and the usage of topical skin barriers can also be put forward. The ability to detect free VX in the bloodstream emerges as one of the significant requirements in toxicokinetics to evaluate the effectiveness of protective means. As a result, VX detection at relatively very low concentrations in small blood quantities (e.g. experiments conducted in mice) is of paramount importance to our study. Previous work pioneered by Reiter et al. [13] has already proposed a complex and elaborate method on how to quantify VX enantiomers in haemolyzed swine blood samples after intravenous or percutaneous supralethal administrations. Herein, we present a new protocol for the quantification of free VX in plasma using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Such a protocol was implemented in our work to quantitatively evaluate the presence of VX in mammalian plasma (small rodents) after skin exposure.

2. Materials and methods

2.1. Chemicals

O-ethyl *S*-[2(diisopropylamino) ethyl] methylphosphonothioate (VX, 98% purity) and O-isobutyl-*S*-[2(diisopropylamino) ethyl] methylphosphonothioate (VR, 95% purity) were both synthesized at the Délégation Générale pour l'Armement – Maîtrise NRBC (Vert-le-petit, France) and used without further purification (see Fig. 1 for their chemical structures).

Ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide (NaOH, 1 mol/L) were supplied by Sigma–Aldrich (Illkirch, France) and Riedel-De Haën (Seelze, Germany), respectively. HPLC grade acetonitrile and absolute grade ethanol were purchased from Sigma–Aldrich. Formic acid (used for mass spectroscopy), *n*-hexane (Picograde, for residue analysis), and diethyl ether (Rectapur) were purchased from Fluka Analytical (Saint Quentin Fallavier, France), LGC Promochem (Wesel, Germany), and Prolabo (Fontenay-sous-

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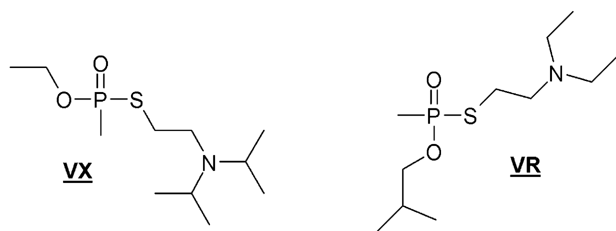

Fig. 1. The chemical structures of VX and VR.

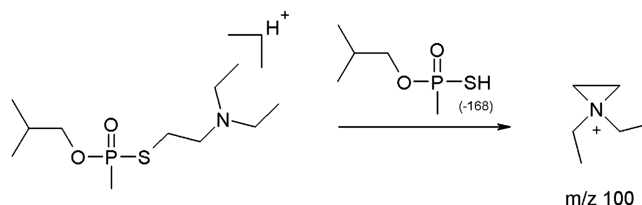

Fig. 3. Fragmentation pathways for protonated VR.

Bois, France), respectively. Panreac chemical company (Lyon, France) provided us with 2-propanol used in instrumental analysis. The water used throughout our experiments was processed by an Alpha Q laboratory water-purification system from Millipore (Molsheim, France). Invitrogen (Cergy-Pontoise, France) supplied us with Hank's Balanced Salt Solution (HBSS, pH 7.4).

2.2. Analytical

2.2.1. Equipment

Chemical analyses were performed using an LC–MS/MS. The chromatographic system (Ultimate 3000 Series, Dionex) consisted of a pump (DGP – 3600M), a stop flow manager (FLM – 3300) and an autosampler (WPS – 3000T) from Voisins le Bretonneux, France. Our chromatographic column consisted of an Allure biphenyl (Restek, Lisses, France) with a length of 100 mm, an internal diameter of 1.0 mm and a particle size of 3 μm.

The detector was a triple-quadrupole mass spectrometer 4000Q Trap from Applied Biosystems (Courtaboeuf, France). A heating/stirring module-evaporating unit (18971 Reacti-Therm and 18780 Reacti-Vap, Pierce) from Montluçon, France and a centrifuge model (4.11C Jouan) (Saint-Herblain, France) were used for sample preparation.

2.2.2. Plasma preparation

Within the framework of our investigations, human blood samples were collected from volunteers, centrifuged for 15 min at 3500 rpm or it was used out-of-date plasma bags from blood donors (Etablissement Français du Sang, Miribelle, France). Plasma was used either fresh or stored at −20 °C and supplemented with EDTA to reach a final concentration of 10 mM [14].

2.2.3. Plasma and internal standards preparation

The work of Reiter et al. [13] was taken as a reference for the VR internal standard. Stock solutions of VX (267 μg/mL) and VR (5 mg/mL) were prepared in isopropanol. All solutions were stored and kept at −20 °C in silanized vials [15]. On the day of the experiment, additional dilutions were done for VR in HBSS to achieve a final concentration of 12.6 ng/mL. Initial dilutions of VX stock solution were processed in HBSS (spiked solution) on the day of the experiment and the volume of the spiked solutions in plasma never exceeds 1% (v/v).

2.2.4. (±) VX extraction from plasma samples

The internal standard solution (8 μL of a 12.6 ng/mL VR solution) was added to spiked plasma (1000 μL) in silanized vials [15]. Then, 55 μL of 1.00 M sodium hydroxide (NaOH) solution were added to the spiked plasma, vortexed for 5 s and fractionized into five vials to have approximately 200 μL of solution in each vial. Thereafter, 300 μL of water were added to each one of them (vials) and vortexed for 5 s followed by the addition of a mixture of 500 μL of diethyl ether/*n*-hexane (25/75, v/v). Then, each vial was vortexed for 10 min and centrifuged at 3200 rpm for 5 min. Following this step, the vials were kept in the freezer at −80 °C for 15 min, then removed from the freezer and placed at ambient temperature for 15 min and centrifuged at 3200 rpm for 5 min. The supernatants collected after centrifugation were transferred to another vial and evaporated with a gentle stream of nitrogen. Finally, the residue was dissolved in 50 μL of a mixture of acetonitrile/formic acid on a 100/0.1 (v/v) ratio and injected into the LC–MS/MS for analysis.

2.2.5. LC–MS/MS analysis

The HPLC system was set to maintain the autosampler temperature at 4 °C and column temperature at 35 °C. A 5 μL aliquot was injected into the liquid chromatography system for analysis. The mobile phase consisted of 0.1% formic acid in acetonitrile (reversed phase) and was kept under isocratic conditions at a flow rate of 40 μL/min. The mass spectrometer (MS) detector was run in positive electrospray ionization with the entrance, declustering, and collision cell exit potentials maintained at 10 V, 61 V and 8 V, respectively. We recorded a value of 29 V for the collision energy and 5400 V for the ion spray voltage. The ion source gas 1 (10 psi), ion source gas 2 (20 psi), curtain gas (35 psi) and collision gas (medium) were all nitrogen and the interface temperature was 200 °C.

The MS was operated in the multiple reaction monitoring mode (MRM) and it was based on monitoring the m/z 268 → 128 transition for VX quantification (more abundant), the m/z 268 → 86 transition for VX confirmation (less abundant) and the m/z 268 → 100 transition indicating the presence of VR (Figs. 2 and 3) [16].

2.2.6. Recoveries, optimized extraction procedure specificity and matrix effect

Extraction recoveries were estimated by comparing peak areas of VX obtained after spiked plasma extraction with those of a standard VX solution prepared in a mixture of acetonitrile/formic acid (100/0.1). The extraction protocol was optimized at 1 ng/mL and the recoveries were determined at three concentration levels notably

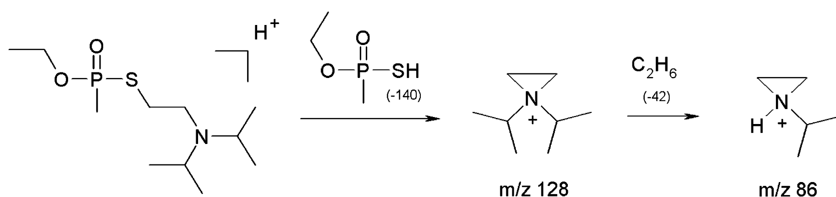

Fig. 2. Fragmentation pathways for protonated VX.

1000 pg/mL (n = 6), 100 and 7 pg/mL (n = 3 in each case). The specificity of the extraction procedure was assessed by comparing blank control plasma with human plasma spiked with VX or internal standard solutions (n = 3). To investigate the matrix effect, human plasma samples (post-prandial samples) from six different healthy volunteers were used (see Section 2.2.2).

The matrix effect due to the plasma matrix was used to evaluate the ion suppression/enhancement in a signal when comparing the post-extracted spiked sample (n = 6, each having been injected 3-fold in the LC–MS/MS system) to the unextracted spiked samples (*i.e.* standard VX solution). It was used to calculate the ratios of the means peak areas in the matrix to those in the standards.

2.2.7. Sensitivity and linearity

The detection limit was determined as the concentration level at which the analyte exhibits a signal-to-noise ratio of 3:1. Furthermore, the quantification limit involved the level at which a signal-to-noise ratio of 10:1 was obtained. The linearity of calibration curves was tested, evaluated, and determined by plotting the ratios of the peak areas (A_{VX}/A_{IS}) against the concentration ratio (C_{VX}/C_{IS}) by means of standard linear regression analysis. According to Sections 2.2.3–2.2.5 such linearity was established with VX spiked plasma samples at a concentration range from 0.5 to 100 pg/mL. Four calibration curves were done and six injections were performed (a set of three in one day and another set of three on different days, over a 3 weeks period) to evaluate the samples stability.

2.2.8. Analytical protocol accuracy

The within-day precision of the present method was determined by the analysis of VX spiked human plasma samples at four different concentration levels (2.5, 10, 50 and 100 pg/mL) and three parallel determinations were made at each level. The between-day precision was also determined in the same manner but on three different days over a 3 weeks period.

Evaluation involving the accuracy of the analytical protocol was based on three concentration levels in plasma samples and they were done at 5, 30, 75 pg/mL (n = 3 each), respectively.

2.3. Animal study

2.3.1. Protocol validation in rodent plasma

2.3.1.1. Methodological adjustments. Since restricted plasma volumes were available, we had to introduce some adjustments regarding the (±) VX extraction protocol mainly by diluting 20 μL of mouse plasma in 480 μL of deionized water in silanized vials [15]. Then, it was added 8 μL of internal standard solution followed by the addition of 8 μL of 1.00 M sodium hydroxide (NaOH) and vortexed for 5 s. 500 μL of diethyl ether/n-hexane (25/75, v/v) were added, vortexed for 10 min and centrifuged at 3200 rpm for 5 min. After a 15 min storage time in the freezer at −80 °C, the vial was removed from the freezer and left outside at ambient temperature for 15 min and centrifuged at 3200 rpm for 5 min. The supernatant collected was transferred to another vial and evaporated with a gentle stream of nitrogen. The residue was dissolved in 50 μL of acetonitrile/formic acid on a 100/0.1 ratio (v/v) and 5 μL was injected into the LC–MS/MS for analysis. In addition to this experiment conducted on 20 μL plasma, conclusive tests were also carried out on 10 μL initial plasma volume diluted in 490 μL of deionized water. Such extraction and chromatographic conditions generated a new range of concentrations between 1 ng/mL and 100 ng/mL. Mouse blood samples were collected into EDTA tubes and immediately processed according to Section 2.2.2.

2.3.1.2. Recoveries, optimized extraction procedure specificity. Extraction recoveries were estimated by comparing peak areas of VX obtained after spiked plasma extraction with those of a standard VX solution prepared in a mixture of acetonitrile/formic acid (100/0.1). The recoveries were determined at three concentration levels notably 100, 50 and 1 ng/mL (n = 3 in each case). The specificity of the extraction procedure was assessed by comparing blank control plasma with mouse plasma spiked with VX or internal standard solutions (n = 3).

2.3.1.3. Linearity and analytical protocol accuracy. The linearity of calibration curves was tested, evaluated, and determined by plotting the ratios of the peak areas (A_{VX}/A_{IS}) against the concentration ratio (C_{VX}/C_{IS}) by means of standard linear regression analysis. According to Sections 2.2.3, 2.3.1.1 and 2.2.5 such linearity was established with VX spiked plasma samples at a concentration range from 1 to 100 ng/mL. Three calibration curves were done and six injections were performed (a set of three in one day and another set of three on different days, over a month) to evaluate the samples stability.

The within-day precision of the present method was determined by the analysis of VX spiked rodent plasma samples at four different concentration levels (1, 10, 50 and 100 ng/mL) and three parallel determinations were made at each level. The between-day precision was also determined in the same manner but on three different days over a month period.

2.3.2. Animals

Six female pathogen-free congenitally athymic, nude mice (Hsd: Athymic Nude-nu, Harlan, France) weighing on average 28.2 ± 1.5 g at the time of the experiment and between 5 and 6 weeks old at the date of the graft, were used. Upon their arrival, the animals were housed separately in an indoor location at standard conditions *i.e.* 24 ± 1 °C on a 12/12 h light/dark cycles under artificial lighting in well-ventilated cages (Seal safe^{TM}) and attached to an IVC rack (From Techniplast, Limonest, France) until they were intoxicated. The pathogen-free air ventilation was maintained to provide a minimum of ten air changes per hour and the animals were allowed to get access to sterilised tap water from the municipal supply and fed *ad libitum* with gamma (γ) irradiated pelleted diet (RM1 (P) VP, SDS, Witham, Essex, England). The animals were acclimatized under these conditions for at least seven days prior to grafting. Dermal exposure to VX was conducted five months after their arrival at the laboratory and on the day of the experiment, each animal was placed in a specially designed metabolic cage.

2.3.3. Experimental protocols

The experiments performed in the present study were approved by the Institutional Animal Care and Research Advisory of the Institut de Recherche Biomédicale des Armées in accordance with the current and appropriate European and French legislation. All experiments conformed to international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering.

2.3.4. Human skin graft on nude mice

To perform surgical procedure (skin surgery) in the animals, isoflurane was used as an anaesthetic agent. General anaesthesia was administered to the animals by inhalation of 3% of isoflurane for 3 min, followed by steady-state inhalation of 1% isoflurane and room air via nose cone. On top of each posterior side of the thorax of every nude mouse, a circular piece of skin of approximately 1 cm in diameter was removed from the panniculus carnosus. This location was chosen so that the mice could hardly eat or scratch the grafted skin [17]. Full-thickness of freshly excised female human skin obtained after abdominal surgery and previously dermatomed to a 484 μm (6% coefficient of variation) thickness was cut into

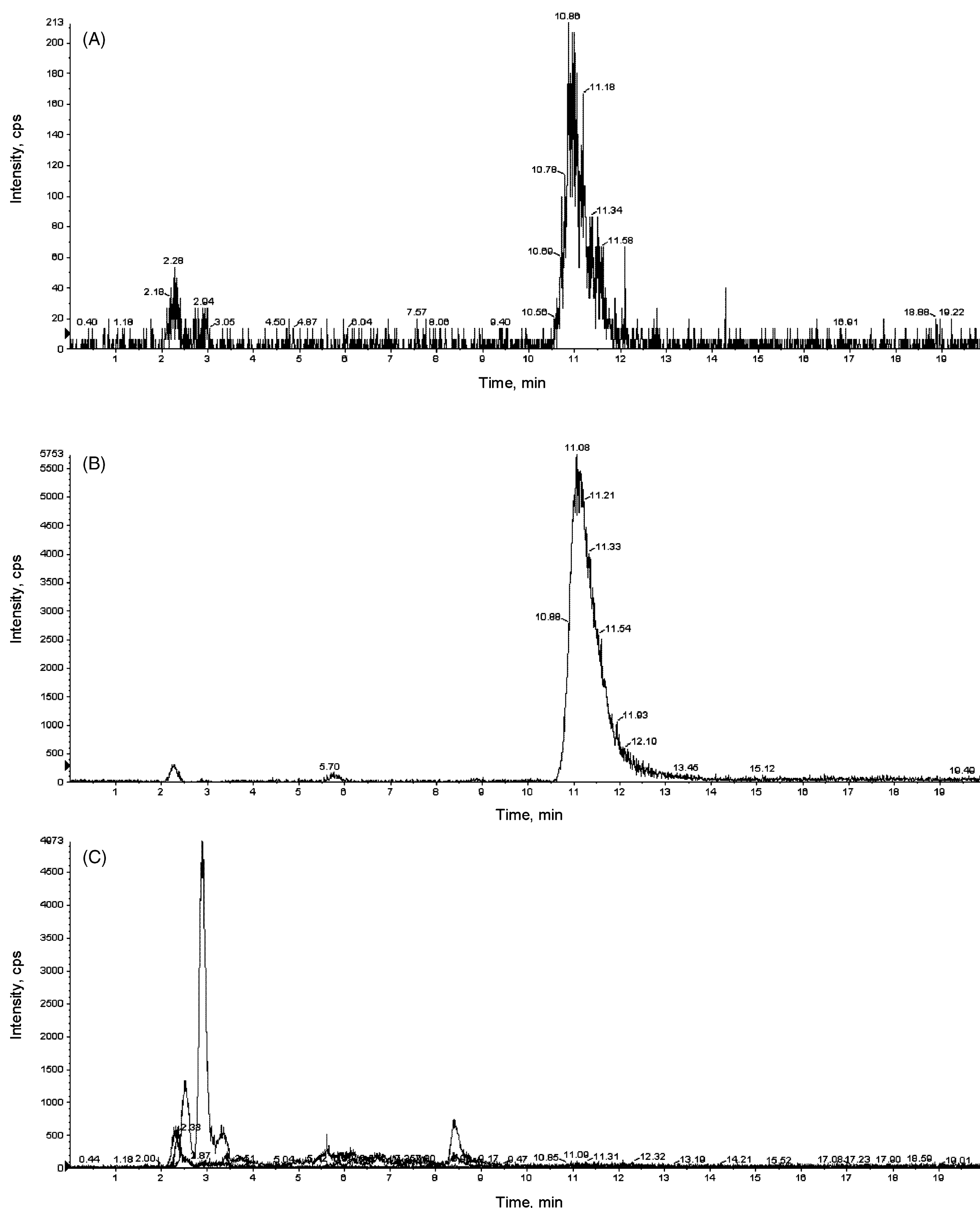

Fig. 4. (A) Chromatogram with m/z 268 → m/z 128 transition for monitoring VX (transition for VX quantification, 2.5 pg/mL) in spiked human plasma sample; (B) chromatogram with m/z 268 → m/z 100 transition for monitoring VR (internal standard, 100 pg/mL) in spiked human plasma sample; (C) chromatogram with m/z 268 → m/z 128 transition for monitoring VX (transition for VX quantification), m/z 268 → m/z 86 transition for monitoring VX (transition for VX confirmation) and m/z 268 → m/z 100 transition for monitoring VR (internal standard) in human plasma blank.

disks of 1.2 cm diameter to fit within the grafted sites (Laboratoires Biopredic, Rennes, France). The human skin was placed in the wound bed and covered successively with a sterile absorbent dressing (Melolin™, Smith and Nephew, Le Mans, France), a tape of Micropore™ (3M, Neuss, Germany), and a 2.5 cm × 4.5 cm elastic bandage (Co-Plus™ LF, BSN medical, Brierfield, England). Each dressing was also protected with a little jacket (Harvard Apparatus, Les Ulis, France) and the graft was allowed to mature and stabilize for 7–8 weeks.

2.3.5. Dermal exposure

A positive displacement pipette (Microman M10, Gilson, Villiers Le Bel, France) was used to deposit VX at the center of each grafted skin. A droplet of 40 μg of VX in 4 μL of HBSS (Invitrogen, Cergy-Pontoise, France) was delivered on each exposure site per animal. Previous work in our laboratory showed that these doses were equivalent to an LD_{100} dose for over an hour which is sufficient to cause severe cholinesterase inhibition in each group.The animals were divided into two groups: an anaesthetized group ($n = 3$)

and a non-anaesthetized one ($n = 3$), in order to present a scenario that closely resembles reality in which collected plasma originated from human whose skin was exposed to VX (with medical care or not). For the first group, to allow a uniform and accurate location of the deposits, one person in protective garments had to immobilize the animals until the VX deposited by another person was no longer visible on the skin surface. In the second group, isoflurane was used under the same conditions during skin deposit and only one person in protective garments carried out all the operations. It is also worthy to mention that in the second group, isoflurane was stopped at $T+20$ min after the deposits when skin surface area of the animal becomes dry. All these operations were performed on a heating carpet to prevent animals' hypothermia. After stopping the inhalation, the anaesthetic effect was quickly neutralized and animals were left conscious for less than a minute at the end of anaesthesia. The experiment was set up to be completed at $T+6$ h and at this time live animals were sacrificed using the cervical dislocation technique. Prior to this time, the other animals showed symptoms of lethal poisoning characterized by salivation, intense tremors, convulsions and finally death due to anoxia resulting from respiratory arrest.

2.3.6. Sampling

Immediately after mice death or euthanasia, heart blood was collected and centrifuged to isolate the plasma from other blood components. Then, plasma samples were collected and supplemented with EDTA (see Section 2.2.2) and kept at −80 °C in a freezer.

3. Results

3.1. Analytical protocol

3.1.1. LC method optimization

First tests conducted were mainly focused on selecting a mobile phase that would optimize VX response during mass spectrometric analysis. At first, we proceeded by testing solvents with different acids in variable quantities. Our compound exhibited optimal signal detection when a mixture of acetonitrile and 0.1% formic acid was used as the mobile phase. The choice of a purely (100%) organic mobile phase to run our polar sample makes the selection of the chromatographic column more complex.

Preliminary tests were done using the Atlantis HILIC Silica (3 μm, 1.0 mm × 50 mm, Waters, Guyancourt, France) and Pepmap C18 (3 μm, 1.0 mm × 150 mm, LC Packings (Dionex), Voisins le Bretonneux, France) columns (available in laboratory). Promising results were obtained with the latter with a major concern regarding the elution time of our compound of interest since it get eluted during dead time. An ideal choice for our column would have similar characteristics with the C18 with an emphasis on correct retention time for VX during mass spectrometric analysis. Restek columns meet the requirements that would give us thin peaks, correct geometry and retention time (Fig. 4). Indeed, using acetonitrile as the mobile phase gives to the biphenyl phase similar retention and selectivity to those of C18 column (mainly hydrophobic interactions) and furthermore it was advised to carry out our analysis using a 100% organic phase.

Table 1
Calculated matrix effect of different human plasma samples ($n = 6$, injected 3-fold on LC–MS/MS).

Different human plasma ($n = 6$)	Matrix effect ± SD
1	0.94 ± 0.023
2	0.91 ± 0.054
3	0.96 ± 0.057
4	0.90 ± 0.065
5	0.98 ± 0.028
6	0.96 ± 0.012

The oven temperature exhibited a significant impact over the quality of the chromatographic peak since an increase in temperature resulted in a better quality of peak.

3.1.2. Extraction yield optimization

3.1.2.1. (±) VX extraction from HBSS samples. Initial optimizations were conducted in HBSS environment (receptor fluid of *in vitro* percutaneous penetration studies). Excellent yields (>99%) were achieved for a single extraction (v/v) and the protocol for (±) VX extraction was carried out as follows: 300 or 500 μL of VX spiked HBSS (1 ng/mL) were placed in silanized vials [15] followed by the addition of 300 or 500 μL (v/v) of diethyl ether (100%). Then, the tube was vortexed for 5 min and centrifuged at 1200 rpm for 5 min. Subsequently, the supernatant was transferred to another vial and evaporated with a gentle stream of nitrogen. The residue was dissolved in a 50 μL mixture of acetonitrile/formic acid (100/0.1, v/v) and injected into the LC–MS/MS.

3.1.2.2. (±) VX extraction from plasma samples. The yield for the plasma samples in HBSS extraction conditions did not exceed 25%. As a result, we had to resort to the protocol described in Section 2.2.4 with the intent to maximize the yield. It is worthy to mention that plasma pH played a crucial role in this regard since a basic pH ($pH = pK_a + 2$; $pH = 11.3$) promotes the extraction of VX from the plasma under such conditions [18]. In addition, diluting plasma with water (200 μL of plasma in 300 μL of water) can also be considered as one of the contributing factors for an increase of the yield. A decrease of the ether level in the extractant did not induce a negative effect on the recovery rate but it upgraded significantly the chromatographic peak quality. An extraction efficiency was achieved within the range of 65% ± 3% at 1000 pg/mL, 63% ± 1% at 100 pg/mL and 70% ± 5% at 7 pg/mL. Those findings convinced us to maintain our extraction protocol since our method is intended to remain adjustable to smaller volumes of samples. Furthermore, the specificity of our extraction method was supported by Fig. 4.

The results of matrix effect, as summarized in Table 1, clearly confirmed the absence of matrix effects considering the few signal suppression as insignificant.

Table 2
Linearity and limits of detection and quantification in VX spiked human plasma.

Investigated change	LCD (pg/mL and fg injected)	LOQ (pg/mL and fg injected)	Reproducibility ($n = 4$)		Repeatability – within-day ($n = 3$)		Repeatability – between-day ($n = 3$)	
			Slope ± SD	Correlation coefficient	Slope ± SD	Correlation coefficient	Slope ± SD	Correlation coefficient
0.5–100 pg/mL	0.5 pg/mL or 50 fg[a] injected	2 pg/mL or 200 fg[a] injected	3.95 ± 0.02	0.9933	4.05 ± 0.12	0.9903	4.01 ± 0.06	0.9961

[a] fg, abbreviation of femtograms.

Table 3
Within-day and between-day variations of VX in human spiked plasma.

Concentration (pg/mL)	Within-day ($n=3$) RSD (%)	Between-day ($n=3$) RSD (%)
2.5	6.2	6.0
10	3.0	6.5
50	6.7	4.2
100	1.8	4.9

Table 4
Summary of accuracy in VX spiked human plasma ($n=3$).

C_{VX} added (pg/mL)	C_{VX} measured (pg/mL) ± SD	Accuracy (%)
40	41.7 ± 0.4	94
5	4.7 ± 0.6	104
2.5	2.9 ± 0.02	116

3.1.3. Sensitivity and linearity

The linearity of the calibration curves was tested and evaluated using the following equation: $A_{VX}/A_{IS} = K \times C_{VX}/C_{IS}$. The six-point calibration curve (2, 5, 10, 50, 75 and 100 pg/mL of VX in human plasma) was linear within a VX concentration range of 2–100 pg/mL. Table 2 shows the sensitivity and stability of the calibration curve with a correlation coefficient greater or equal to 0.9903 indicating a good linearity.

3.1.4. Accuracy

As can be seen from Table 3, the within-day and between-day precisions (presented as relative standard deviation, RSD) were less than 6.7 and 6.5%, respectively.

Table 4 provides evidence of high level of accuracy generated through our methodology.

3.2. Animal study

3.2.1. Results of protocol validation

3.2.1.1. Extraction yield. An extraction efficiency was achieved within the range of 73% ± 3% at 100 ng/mL, 54% ± 8% at 50 pg/mL and 65% ± 5% at 1 ng/mL. Furthermore, the specificity of our extraction method was supported by Fig. 5.

3.2.1.2. Linearity and accuracy. The linearity of the calibration curves was tested and evaluated using the following equation: $A_{VX}/A_{IS} = K \times C_{VX}/C_{IS}$. The five-point calibration curve (1, 5, 10, 50 and 100 ng/mL of VX in mouse plasma) was linear within a VX concentration range of 1–100 ng/mL. Table 5 shows the stability of the calibration curve with a correlation coefficient greater or equal to 0.9974 indicating a good linearity.

As can be seen from Table 6, the within-day and between-day precisions (presented as relative standard deviation, RSD) were less than 5.0 and 3.5%, respectively.

3.2.2. Results of in vivo application

A six-point calibration curve (1, 2, 3, 5, 7 and 10 ng/mL of VX in mouse plasma) was linear within a VX concentration range of 1–10 ng/mL with response being defined as $A_{VX}/A_{IS} = K \times C_{VX}/C_{IS}$. The data show an average slope of 2.13 ± 0.03 and good correlation ($R^2 = 0.9927$).

Table 6
Within-day and between-day variations of VX in mouse spiked plasma.

Concentration (ng/mL)	Within-day ($n=3$) RSD (%)	Between-day ($n=3$) RSD (%)
1	4.4	0.9
10	5.0	2.6
50	2.9	3.5
100	2.4	3.1

Measured concentrations for plasma free VX ranging from 1.5 to 2.5 ng/mL are presented in Table 7. Fig. 5 shows chromatogram of a VX plasma sample (intoxicated mice).

Concerning the possible effects of anaesthesia administered to animals, results collected for non-anaesthetized animals show greater data dispersion with respect to post-intoxication survival expectancy. In fact, an animal from the non-anaesthetized group was euthanized 6 h after intoxication whereas the other two animals experienced a life expectancy lasting for approximately 1 h and a half. Indeed, considering the first significant intoxication signs in rats, e.g. brief episodes of convulsions observable within the first hours post-VX exposure, non-anaesthetized animals showed their symptoms earlier than those from the isoflurane treated group. Furthermore, the anaesthetized subjects seemed to live longer than the non-anaesthetized ones.

4. Discussion

The present study has two major aspects: to contribute an innovative protocol for free VX quantification in plasma and to quantify VX in small rodents. Regarding our analytical protocol, we developed a time-saving and reliable method for VX quantification in spiked plasma using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Our distinctive method allowed us to reach a detection limit of 50 fg injected (0.5 pg/mL). Comparatively, in earlier experiments dedicated to (±) VX determination in blood and plasma, van der Schans et al. [18] and McGuire et al. [19] reached a detection limit of 0.8 pg (≈32 pg/mL) and 0.4 pg (≈330 pg/mL) injected, respectively, *i.e.* values that are 8–16 times higher than our detection limit.

The distinctive degree of efficiency within our methodology is based on the following combination of assets namely VX extraction recovery performance levels and methodological accuracy. VX saline extraction recovery averaged 99% *i.e.* a value in complete agreement with earlier findings released by McGuire et al. [19] but yet above those reported elsewhere [13,18]. Taking into account our experimental conditions, VX extraction recovery rate in plasma (65%) is slightly below the 75% recovery rate achieved by Reiter et al. [13] from haemolyzed blood samples. At last, our method accuracy is also in agreement with theirs [13].

A cross-sectional analysis combining extraction steps, solvent consumption and retention times provided substantial evidence with regard to time and implementation efficiency assets generated by our methodological protocol. This fact is supported by our saline extraction technique (single extraction) that provided easier implementation conditions as compared to McGuire's [19] practice that involved a two-step extraction and pH control. Although our

Table 5
Linearity in VX spiked mouse plasma.

Investigated range	Reproducibility ($n=3$)		Repeatability – within-day ($n=3$)		Repeatability – between-day ($n=3$)	
	Slope ± SD	Correlation coefficient	Slope ± SD	Correlation coefficient	Slope ± SD	Correlation coefficient
1–100 ng/mL	2.56 ± 0.02	0.9976	2.79 ± 0.19	0.9974	2.45 ± 0.08	0.9992

Table 7
Results of the intoxication of mice by VX. Comparison between anaesthetized and non-anaesthetized mice.

Time between deposit and clinical signs of intoxication		Time between deposit and death	C_{VX} in plasma (ng/mL)
Anaesthetized group (n = 3)			
1	3 h:00	3 h:25	1.63
2	2 h:00	2 h:21	1.57
3	1 h:40	2 h:18	2.63
Non-anaesthetized group (n = 3)			
1	1 h:00	1 h:16	2.71
2	2 h:00	Euthanasia – 6 h	0.16
3	1 h:20	1 h:51	1.57

plasma extraction process proved more complex than our saline extraction, it nevertheless required significantly smaller solvent and reagent quantities when compared to the one described by Reiter et al. [13] As an example, for 2 mL of blood, the latter used approximately 200 mL of solvent or reagent whereas our method required only 5 mL of solvent and reagent for 1 mL of plasma. In addition, our findings exhibited satisfactory retention times of 11 min.

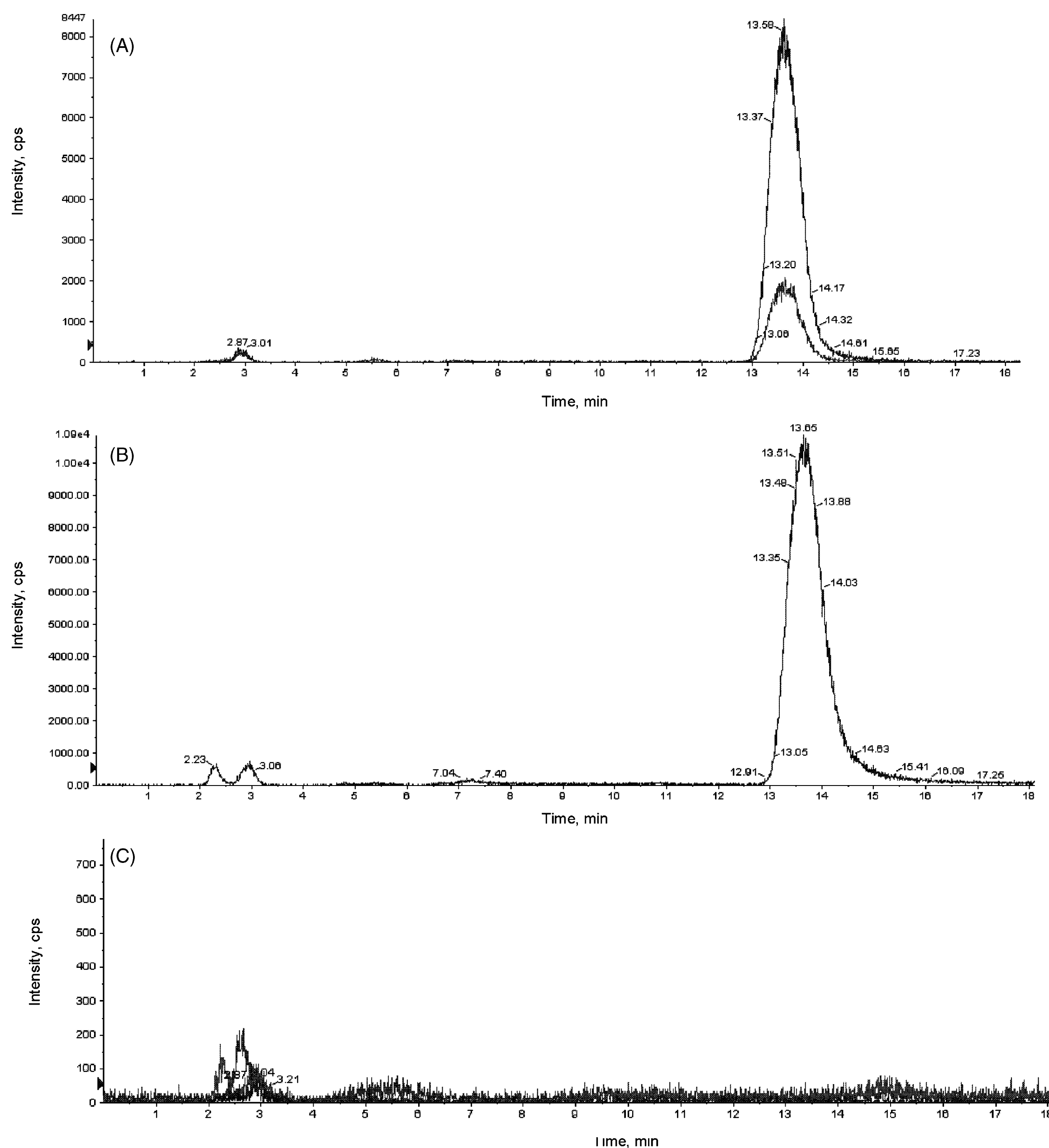

Fig. 5. (A) Chromatogram with m/z 268 → m/z 128 transition for monitoring VX (transition for VX quantification, 1.57 ng/mL) and m/z 268 → m/z 86 transition for monitoring VX (transition for VX confirmation) in VX plasma sample (intoxicated mice); (B) chromatogram with m/z 268 → m/z 100 transition for monitoring VR (internal standard, 0.1 ng/mL); (C) chromatogram with m/z 268 → m/z 128 transition for monitoring VX (transition for VX quantification), m/z 268 → m/z 86 transition for monitoring VX (transition for VX confirmation) and m/z 268 → m/z 100 transition for monitoring VR (internal standard) in mouse plasma blank.

Our experiment with VX intoxicated rodents (mice) proved that anaesthetized animals seem to live longer than the non-anaesthetized subjects. The tachycardia induced by the stress of mechanical restraint may be responsible for a faster dissemination of VX. Results and subsequent conclusions reported by previous authors [20–22] were usually related to the anaesthetized animals (situation more acceptable for the animal and the operator) but rarely reflected what was actually occurring in the acutely poisoned unanaesthetized subjects. As a result, additional experimentation should be conducted to deepen data.

The mouse that was non-anaesthetized and euthanized showed similar clinical signs at the same moment as the other two non-anaesthetized mice. Moreover, human skin grafts originated from the same donor showed similar characteristics as the other two at post-mortem examination. Considering the conditions of dermal exposure (see Section 2.3.4), it can be ruled out that such a discrepancy is attributed to an inaccurate deposition of VX on the grafted human skin. Consequently, the difference of survival time and rate of free VX in plasma (see Table 5) between the euthanized mouse and the other two could be seen as a result of an intra-species variation of organophosphorus hydrolase rate and of the toxicokinetic of VX [23].

Plasma samples have been collected when clinical signs were most severe in the animals. All measured concentrations of plasma free VX in intoxicated mice varied from 1.5 to 2.5 ng/mL. This concentration range is roughly in agreement with Reiter's results derived from swine blood following $3\times$ LD_{50} doses of undiluted VX [13] administration. Thus, our distinctive methodology is suitable for VX determination in small rodents inasmuch as the present protocol could be applied to 20 μL blood sampling.

5. Conclusion

Our distinctive methodological approach to quantify VX in plasma allowed us to reach a detection limit below 1 pg/mL for a 5 μL-injected quantity. Our results presented herein, provide substantial evidence regarding the relevance of the technique that was used to extract relatively small amount (between 10 and 1000 μL) of plasma in rodents. The next goal of our research will focus on VX enantiomer discrimination. To the best of our knowledge, Reiter et al. [13] have performed such a separation and using VX intoxicated swine they demonstrated that the pharmacokinetics of blood VX enantiomers depend on the poisoning mode (route of exposure) affecting the animal. If such an observation is still valid in our model, human skin grafted onto nude mice, a more thorough investigation within the field of percutaneous intoxications warrants further scrutiny.

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