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Quantification of nerve agent adducts with albumin in rat plasma using liquid chromatography–isotope dilution tandem mass spectrometry

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ABSTRACT

A sensitive method for the determination of the organophosphorus nerve agents sarin, soman and VX adducts with tyrosine residue of albumin in rat plasma has been developed and validated using liquid chromatography–isotope dilution tandem mass spectrometry (LC–IDMS/MS). *O*-(*O*-Alkyl methylphosphonyl) tyrosine adducts and their deuterated products that were used as the internal standards were synthesised to establish the quantitative isotope-dilution method. Protein purification and solid-phase extraction (SPE) were applied to improve the recovery efficiency, reduce interference and achieve high sensitivity. The method provided a detection limit of 0.01 ng/mL for sarin and soman adducts and 0.05 ng/mL for the VX adduct. The value of the intra-day relative standard deviation over the calibration range was less than 6.16% ($n = 6$), and that of the inter-day was less than 12.7% ($n = 6$). The recovery varied from 86% to 111%. This sensitive method was successfully applied to the analysis of adducts in rat plasma after nerve agent exposure, and the results demonstrated the dose–effect relationships.

1. Introduction

Organophosphorus nerve agents (OPs) are a type of chemical warfare agent with high toxicity that can inhibit the enzymatic activity of cholinesterase (ChE). Although the use, production and stockpiling of nerve agents are prohibited by the Chemical Weapons Convention (United Nations 1993), their illegal use in terrorist attack and warfare is still a threat all over the world. For example, sarin has been used both in the terrorist attacks in the Tokyo subway and Matsumoto City in the 1990s [1,2]. VX was used for an assassination in Japan. Tabun was used as a chemical weapon in the Iraq–Iran conflict in the 1980s [3].

Nerve agents are distributed and metabolised rapidly *in vivo* after exposure, existing partly as the intact agent, partly as degradation products or metabolites and partly as covalently bound to macromolecules such as enzymes and other proteins [4]. These three types of compounds could be used as *in vivo* biomarkers of nerve agent exposure. The analysis of biomarkers in biomedical samples such as blood and urine plays an important role in forensic investigations of the alleged use of nerve agents. Detection of biomarkers not only can provide forensic evidence of exposure in the event of military or terrorist use of nerve agents but also can be used for diagnostic purposes to ensure appropriate medical treatment. Biomarkers also can be used to monitor exposure levels in workers engaged in activities such as demilitarisation.

The ideal biomarker should be present in a readily obtainable matrix such as blood or urine, have a relatively long lifetime, and provide an unequivocal identification of the agent. The nerve agents are eliminated rapidly in the body. The intact agents, themselves, as biomarkers are inappropriate because they cannot be easily detected unless the sample was collected very soon after the exposure. Metabolism of nerve agents is dominated by hydrolysis, and the free metabolites occur primarily in urine. The urinary excretion may be accounted for up to ~90% of an absorbed dose of agent, and up to ~90% of it is usually eliminated within the first 48–72 h following exposure [5]. Covalent adducts of nerve agents with blood proteins have a longer lifetime than free metabolites and may exist in blood for up to several weeks post-exposure [6].

Currently, the measurement of ChE activity in blood by the colorimetric Ellman procedure [7] is generally used to diagnose poisoning by nerve agents. Because of the low specificity and the unreliable evidence for diagnoses at inhibition levels less than 20%, the method is only used to preliminarily identify the poisoning. The method based on measurement of the hydrolysis products of nerve agents such as *O*-alkyl methylphosphonic acids has also been developed for the verification of nerve agents [8]. The hydrolysis products are excreted mainly in urine, which is easier to obtain, simpler to prepare and more variable than blood. However, the *in vivo* rapid elimination rate of the hydrolysis products limits the use of this method for retrospective detection of exposure.

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Analysis of nerve agent adducts with proteins in blood is an alternative approach. The major advantage of protein adducts is their much longer lifetime as compared to that of hydrolysis products, which makes them suitable for retrospective assaying. The biochemical targets of nerve agents are the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), whose activity is inhibited by the agents; the active site is the serine-203 residue for AChE [9] and the serine-198 residue for BuChE. Fluoride reactivation [10,11] in which the organophosphorus moiety is displaced as a fluoridate is currently the most sensitive method for measuring cholinesterase (ChE) inhibition by nerve agents, which is available at <1% inhibition rate of BuChE. However, because of the *O*-dealkylation of the phosphoryl moiety, a process known as "aging", the method cannot be used for the validation of soman exposure. Adducts with ChE can also be confirmed by the peptides containing the phosphonyl moiety that are produced by digestion with an appropriate protease such as pepsin [12,13]. Because of the loss of structural information that results from aging, this approach may not be appropriate for unequivocal analysis depending on the extent of aging. Another disadvantage of the method results from the possibility of replacement of the organophosphorus moiety by oximes in the body of a poisoned person under medical treatment [14].

Nerve agents can also react with the tyrosine residue of albumin in blood and produce the adducts that are measured as the phosphylated peptides or amino acids after protease digestion. Recent studies demonstrated that the phosphylated residue on human albumin is tyrosine-411 when nerve agents bind to albumin [14,15]. A LC–MS/MS method based on the phosphylated tyrosine has been established and used for the determination of the soman and tabun adducts in guinea pigs, demonstrating that these adducts can be detected up to 7 days after exposure and oxime therapy [14]. The results indicated that the tyrosine adducts were an appropriate biomarker for the retrospective detection of nerve agent exposure because of their relatively long lifetime and the fact that they are less influenced by oxime therapy. This result is verified in subsequent study of same group [16].

In this paper, we present a newly developed sensitive and accurate quantitative method for the determination of *O*-(*O*-alkyl methylphosphonyl) tyrosine adducts in blood samples using pronase digestion and liquid chromatography–isotope dilution tandem mass spectrometry (LC–IDMS/MS). The HiTrap™ Blue affinity column was chosen to isolate and purify albumin from rat plasma that was exposed to nerve agents, and the solid-phase extraction (SPE) pretreatment procedure was also optimised in detail. *O*-(*O*-Alkyl methylphosphonyl) tyrosine adducts and their deuterated products, which were used as the internal standards, were synthesised. The method was validated and applied to the analysis of adducts in blood samples collected from rats following exposure to nerve agents, the result of which revealed the relationship between the exposure dosage of agents and the amount of adducts.

2. Experimental

2.1. Caution

Nerve agents are highly toxic. Handling of the agents by experienced personnel was performed in a well-ventilated cupboard. Gloves and stringent protective measures were utilised.

2.2. Chemicals and materials

Sarin (GB), soman (GD) and VX were obtained from the Institute of Chemical Defence of China, and the purity of each was analysed by means of GC and GC–MS. Pronase (protease type XIV from *Streptomyces griseus*. EC3.4.21.4) and S-butyrylthiocholine iodide were purchased from Sigma–Aldrich (US). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Fluka (Switzerland). Formic acid, N-carbobenzyloxy-L-tyrosine benzyl ester and *O*-phospho-L-tyrosine (P-tyrosine) were purchased from Acros (Belgium). Ethanol-d_6 and isopropyl alcohol-d_8 (D-enrichment ≥98.5%) were purchased from Acros (Belgium). Benzyl bromide was purchased from J&K Chemical (US) and 1,8-diazobicyclo [5.4.0] undec-7-ene was purchased from Aldrich (US). Acetonitrile was purchased from J.T. Baker (US). The HiTrap™ Blue HP affinity columns (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) were purchased from GE Healthcare (UK), the ultra-filtrated tube from Millipore (US) and C_{18} sorbents from Alltech (US). Methylphosphonic dichloride was synthesised in our lab, with the purity as high as 98%. Water was purified using a Milli-Q system (Millipore).

The flow-injection pump was a KDS-100CE model from KdScience (US), and the centrifugal evaporator (RVC 2-33 CD Plus) was purchased from Christ.

2.3. Standards and spiking solutions

Stock solutions of GD, GB and VX (10 mg/mL) for *in vivo* experiments were prepared in acetonitrile and stored for up to 1 month at −20 °C in glass vials. The stock solutions were further diluted in isopropanol prior to final dilution in normal saline (spiking solution) on the day of the experiment.

2.4. Instrumentation

The analytical LC–MS/MS for the quantification of nerve agent-tyrosine adducts consisted of a 1200 LC and a 6430 triple quadrupole mass spectrometer (Agilent, USA).

2.5. Synthesis of O-(O-alkyl methylphosphonyl) tyrosine adducts and the corresponding deuterated adducts

2.5.1. O-Isopropyl, O-ethyl and O-pinacolyl methylphosphonochloridates

The *O*-alkyl methylphosphonochloridates were prepared by the controlled addition of the appropriate alcohol to methylphosphonic dichloride [17]. The alcohol was added slowly into the methylphosphonic dichloride solution in DCM. An ice-water bath was used to control the velocity of the reaction, especially for the ethyl alcohol. The reaction process was monitored *via* GC–MS. The reaction system was washed with water, dried with Na_2SO_4 and then filtered. The deuterated *O*-alkyl methylphosphonochloridates were prepared by the same method, with the replacement of ethyl and isopropyl alcohol by ethanol-d_6 and isopropyl alcohol-d_8.

2.5.2. N-carbobenzyloxy-L-tyrosine benzyl ester

N-carbobenzyloxy-L-tyrosine benzyl ester was prepared from N-carbobenzyloxy-L-tyrosine and benzyl bromide according to the method of Kitas [18].

1,8-Diazobicyclo [5.4.0] undec-7-ene (1.52 g, 10 mmol) and benzyl bromide (1.71 g, 10 mmol) were added to a suspension of N-carbobenzyloxy-L-tyrosine in THF (40 mL) and refluxed for 1.5 h. The by-product was filtered off, and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (40 mL), washed with 1 M HCl (2× 20 mL), 5% $NaHCO_3$ (2× 20 mL) and saturated NaCl (20 mL), dried with Na_2SO_4 and then filtered. The solvent was removed under reduced pressure, and a yellow sticky liquid was obtained. Recrystallization of the products was

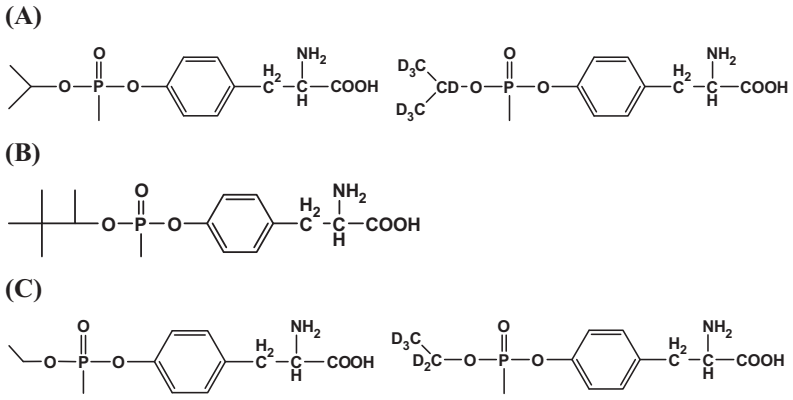

Fig. 1. Chemical structural formulae of synthetic nerve agent adducts with tyrosine and their deuterated internal standards: (A) sarin-tyrosine adduct and D7-sarin-tyrosine adduct, (B) soman-tyrosine adduct, and (C) VX-tyrosine adduct and D5-VX-tyrosine adduct.

performed from ethyl acetate/petroleum ether (b.pt. 60–90 °C) and gave yellow needles.

2.5.3. Protected phosphonylated tyrosine

O-(*O*-Alkyl methylphosphonyl) tyrosine analogues (alkyl = ethyl, [C_2D_5] ethyl, isopropyl, [C_3D_7] isopropyl and pinacolyl) were prepared by the method of Williams [14], with a few modifications. A suspension of N-carbobenzyloxy-L-tyrosine benzyl ester (0.472 g, 1.06 mmol) in acetonitrile (10 mL) was stirred and cooled in an ice-water bath while sodium hydride (80 mg, 2 mmol) was added, then stirred for an additional 30 min. The mixture was allowed to warm to room temperature and then was stirred for 1 h. Next, the appropriate *O*-alkyl methylphosphonochloridate was added dropwise, and the mixture was stirred for another 3 h. The reaction was monitored by thin-layer chromatography (silica gel) until there was only a single component. The solvent was removed using a rotary evaporator; the water was then added, and the product was extracted into chloroform (2× 20 mL). The chloroform extract was washed with water (10 mL), dried with anhydrous Na_2SO_4, and concentrated using a rotary evaporator. The residue was passed through a silica gel chromatography column using petroleum ether (b.pt. 60–90 °C) (70%, v/v) – acetone (30%, v/v) as the eluent.

2.5.4. O-(O-Alkyl methylphosphonyl) tyrosine

The benzyl and N-carbobenzyloxy protecting groups of the products were removed by catalytic hydrogenation. Palladium and 95% ethanol were used as the catalyst and solvent, respectively. After stirring at room temperature overnight under an atmosphere of hydrogen, the reaction mixture was centrifuged and concentrated to a colourless, viscous oil, which contained the tyrosine adducts with purity >90% by HPLC and LC/MS. The product was also characterized based on ^{1}H NMR and mass spectral data. The chemical structure of synthetic nerve agent-tyrosine adducts and their deuterated internal standards are shown in Fig. 1.

2.6. Animals, in vivo experiments and blood sampling

2.6.1. Animals

Adult male Sprague-Dawley rats weighing ~150 g were purchased from the Laboratory Animal Center of Beijing, and all of the animals were SPF (Specific Pathogen Free) grade. The rats were allowed to acclimate for at least one week prior to experimental use.

2.6.2. Nerve agent administration

Nerve agents (GB, GD and VX) were administered by subcutaneous injection in a total volume of 400 μL. Each nerve agent was diluted in isopropanol prior to a final dilution in saline immediately before administration. The final concentration of isopropanol was less than 1%. Doses of nerve agents were chosen as: sarin for 9–36 μg/kg, soman for 24–96 μg/kg and VX for 8 μg/kg.

2.6.3. Blood sampling

The animals were anesthetised by administering a 20% ethyl carbamate solution as an intraperitoneal injection 48 h after injection of the agents, then the blood samples were obtained from the abdominal aorta in 5 min. The rat would go to death after 8–10 mL of blood was collected. Samples were collected into heparin sodium tubes and immediately centrifuged at 4000 rpm for 15 min to isolate the plasma and then were frozen and stored at −80 °C until analysis.

2.7. Sample preparation

2.7.1. Determination of BuChE activity

BuChE activity was measured spectrophotometrically using a modified Ellman assay [7]. The mixture solution (2.75 mL) consisted of 50 μL of rat plasma, 100 μL of 10 mM S-butyrylthiocholine iodide as the substrate and 100 μL of 10 mM DTNB as the chromogen in 0.1 M phosphate buffer (pH 8.0). The activity was determined in a week after sample collection, and the assay was performed at room temperature.

2.7.2. Isolation of albumin from rat plasma

Rat plasma (0.5 mL) was applied to a HiTrap™ Blue HP affinity column that was incorporated with a flow injection pump after conditioning with buffer A (5 mM NaH_2PO_4 and 15 mM Na_2HPO_4, pH 7, 10 mL). The column was washed with buffer A (10 mL) and then eluted with buffer B (2 M NaCl in buffer A, pH 7, 10 mL). The column was regenerated by washing with buffer A (12 mL).

2.7.3. Digestion of albumin with pronase

The eluate of buffer B was filtered using a 30-kDa cutoff ultrafilter tube, and the retentate was washed with 50 mM NH_4HCO_3. Then, the retentate was transferred to a 1.5 mL EP tube, and the filter tube was rinsed with 200 μL of 50 mM NH_4HCO_3. The rinse fluid was combined with the retentate. Pronase solution (200 μL of a 10 mg/mL solution in 50 mM NH_4HCO_3) was added to the retentate of albumin. After incubation for 6 h at 37 °C, the incubation mixture was centrifuged and subjected to SPE.

2.7.4. SPE procedure

The SPE procedure was optimised using an albumin-pronase digestion solution spiked with same concentrations of various

Table 1
Solid-phase extraction (SPE) conditions for isolation of nerve agent-tyrosine adducts from rat albumin digests.

Compounds	SPE sorbent (250 mg, 1.5 mL)	Conditioning	Wash solvent	Elution solvent
Sarin-tyrosine adduct	C_{18}	ACN (3× 1 mL) H_2O (3× 1 mL)	H_2O (3× 1 mL)	10% ACN aqueous solution (3× l mL)
Soman-tyrosine adduct	C_{18}	ACN (3× 1 mL) H_2O (3× 1 mL)	H_2O (3× 1 mL)	60% ACN aqueous solution (3× 1 mL)
VX-tyro sine adduct	C_{18}	ACN (3× 1 mL) H_2O (3× 1 mL)	H_2O (3× 1 mL)	10% ACN aqueous solution (3× l mL)

synthetic nerve agent-tyrosine adducts and their deuterated products. The SPE cartridge was packed with 250 mg of C_{18} sorbent per cartridge. 10–60% ACN aqueous was used as the elution solvent, the efficiency of which was compared utilising LC–MS/MS. The optimised SPE conditions for nerve agent-tyrosine adducts are summarized in Table 1.

After elution, the samples were concentrated to dryness at 50 °C using a centrifugal evaporator and the residues were redissolved in Milli-Q water (100 μL) for LC–IDMS/MS experiments.

2.8. LC–IDMS/MS analysis

The separation was performed on an Agilent 1200 HPLC, and the HPLC column employed was a 15 cm × 2.1 mm Waters XTerra MS C_{18} with 3.5 μm particles. The mobile phase consisted of 0.05% formic acid and 5% acetonitrile in water (solvent A) or 0.05% formic acid and 5% water in acetonitrile (solvent B). The injection volume was 5 μL. A linear gradient elution was performed at a flow rate of 0.25 mL/min, with a gradient as described in Table 2.

The mass spectral analysis was performed on an Agilent 6430 triple quadrupole mass spectrometer controlled by MassHunter software. The mass spectrometer was operated in positive-ion, multiple reaction monitoring mode. The specific operational settings used were the following: 350 °C gas temperature, gas flow rate of 8 L/min, nebulizer gas at 35 psig, 3800 V VCap, 65 V skimmer1, 750 V Oct and the Dwell was 100.

2.9. Method validation

The method was validated with regard to specificity, recovery, precision, linearity and sensitivity.

2.9.1. Study of specificity

The specificity of the LC–IDMS/MS method was checked by analysing rat plasma samples of six blanks and six samples spiked with adducts. The plasma samples from rats exposed to nerve agents were also investigated. The chromatograms of blank and spiked samples were compared to chromatograms from synthetic adducts standard solutions.

2.9.2. Recovery

The recoveries of sarin, soman and VX adducts with tyrosine were assessed at three concentration levels of 1, 10 and 100 ng/mL. The matrix was prepared as described in Sections 2.7.2 and 2.7.3 before the SPE procedure was conducted, except that rat plasma was not exposed to nerve agents. Accurate quantities of synthesised sarin, soman and VX adducts and their internal standards were then added into the matrix, which was followed by SPE steps. Meanwhile, an equivalent amount of synthesised adducts were added into the SPE cleaned blank matrix. After analysis by LC–IDMS/MS, the recoveries were calculated by comparing the peak area ratios.

Table 2
Linear gradient elution conditions for nerve agent-tyrosine adducts.

Compounds	Gradient
Sarin-tyrosine adduct	5%B (0–2 min) to 25%B (2.1–6 min) to 90%B (7–10 min)
Soman-tyrosine adduct	10%B (0–2 min) to 35%B (2.1–6 min) to 90%B (7–10 min)
VX-tyrosine adduct	5%B (0–2 min) to 25%B (2.1–6 min) to 90%B (7–10 min)

2.9.3. Precision

The intra-day variation was determined by analysing the six replicates on the same day, and the inter-day variation was determined on six consecutive days. The precision of the analytical procedure was tested at three concentration levels (1, 10, 100 ng/mL; $n = 6$ for each). The matrix of the sample was produced from rat plasma (0.5 mL), which was prepared using a HiTrap™ Blue column, digested by pronase and then cleaned with SPE procedure. The relative standard deviation (RSD) was taken as a measure of precision.

2.9.4. Linearity and sensitivity

Linearity calibration curves were generated within the final concentration range of 0.1–1000 ng/mL for sarin adduct, 0.5–1000 ng/mL for soman adduct and 0.1–160 ng/mL for VX adduct by spiking synthetic adducts in a plasma-albumin-pronase digestion matrix, which is same as Section 2.9.3. The internal standards were also added into the spiked matrix with the final concentrations of 60 ng/mL for sarin, 50 ng/mL for soman and 9.2 ng/mL for VX. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined in the same matrix at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

2.9.5. Adducts quantification in rat plasma samples

Adduct concentrations in rat plasma samples were determined by LC–IDMS/MS and were referred to calibration curves of plasma-albumin-pronase digestion mixture samples spiked with adduct (0.1–1000 ng/mL) and internal standards (60 ng/mL for sarin, 50 ng/mL for soman and 9.2 ng/mL for VX). Deuterated *O*-(*O*-isopropyl methylphosphonyl) tyrosine and deuterated *O*-(*O*-ethyl methylphosphonyl) tyrosine were used as internal standards in the quantitative detection of sarin and VX adducts, respectively. Because deuterated pinacolyl alcohol is not commercially available in China, the deuterated *O*-(*O*-pinacolyl methylphosphonyl) tyrosine cannot be conveniently synthesised. *O*-Phospho-L-tyrosine (P-tyrosine) was selected for use as an internal standard for the quantification of the soman adduct.

2.10. Data analysis

The data were analysed using Mass Hunter Quantitative Analysis workstation software from Agilent. Regression and calculation were also performed with this software package. The chromatographic data were smoothed once prior to integration and fitted by linear regression using $1/x$ for both axes.

3. Results and discussion

3.1. Synthesis of O-(O-alkyl methylphosphonyl) tyrosine adducts

In the preparation of *O*-alkyl methylphosphonic chloridates, methylphosphonic dichloride reacted directly with the appropriate alcohol. The optimised synthesis procedure was simpler and safer than the reported methods [16]. The reactions were

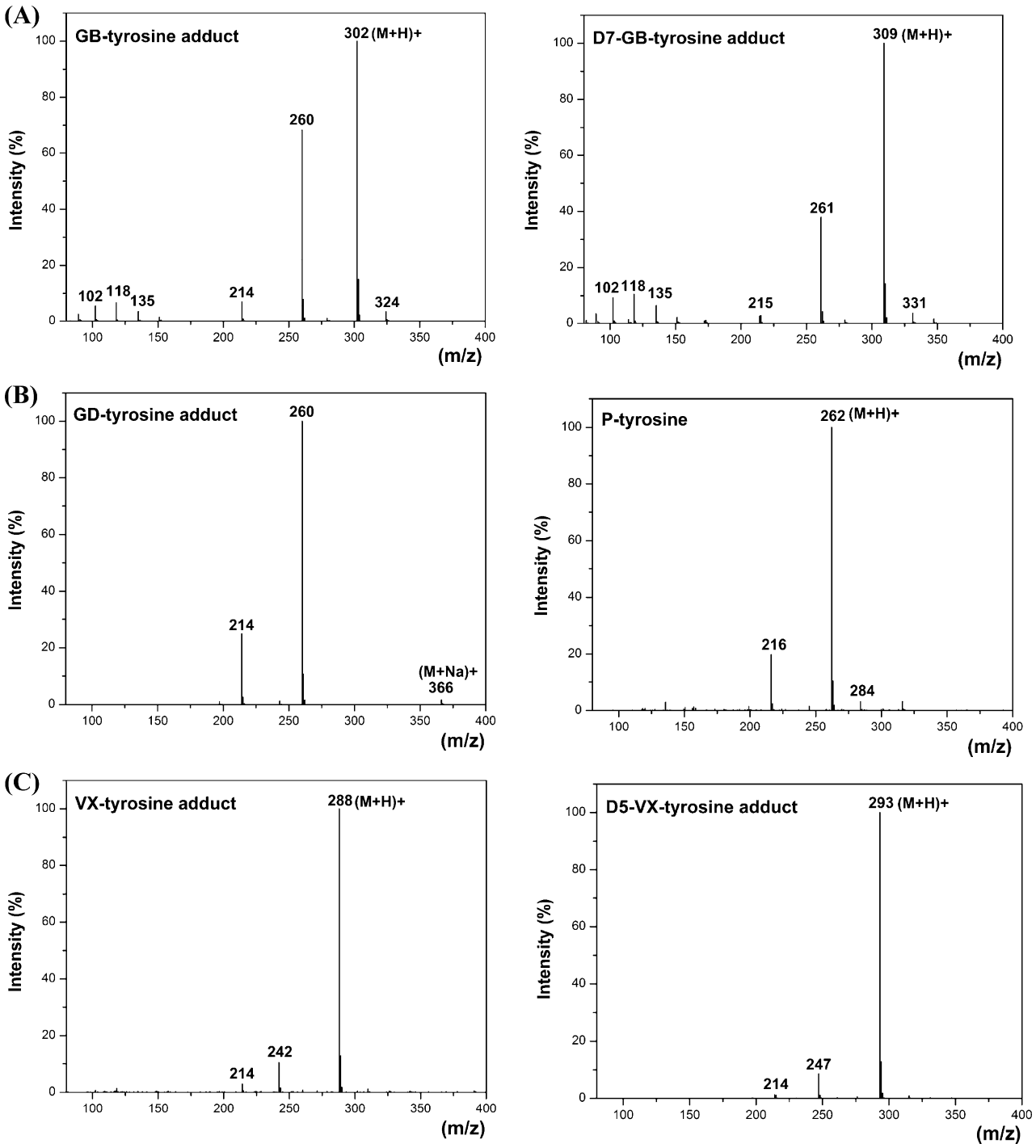

Fig. 2. ESI-MS spectra for the organophosphylated tyrosine adducts and their deuterated internal standards: (A) sarin-tyrosine adduct and D7-sarin-tyrosine adduct, (B) soman-tyrosine adduct and P-tyrosine, (C) VX-tyrosine adduct and D5-VX-tyrosine adduct.

monitored by GC–MS to reduce the production of the dialkyl methylphosphonate. The reaction time was different for various alcohols because of the steric hindrance, and the sequence is pinacolyl > isopropyl > ethyl.

Anhydrous ethanol was used as the solvent of catalytic hydrogenation, whereas the hydroxyl group of tyrosine was esterified easily by the solvent. In our modified method, 95% ethanol was used as the solvent, and the esterification was greatly reduced. The LC–IDMS/MS mass spectrum of synthetic nerve agent-tyrosine adducts and their deuterated internal standards are shown in Fig. 2.

3.2. Sample preparation

Generally, pronase was used to digest the exposed plasma directly [14]. Our previous *in vitro* experiment showed that the isolation of albumin from plasma was favourable to achieve higher digestion efficiency, lower interference from other proteins and lower detection limits. The HiTrap™ Blue column is a very versatile tool in the proteomics strategy for the removal of many high-abundance proteins such as albumin, so its advantages were utilised to isolate the albumin from rat plasma in our research. The results showed that the *O*-(*O*-alkyl methylphosphonyl) albumin

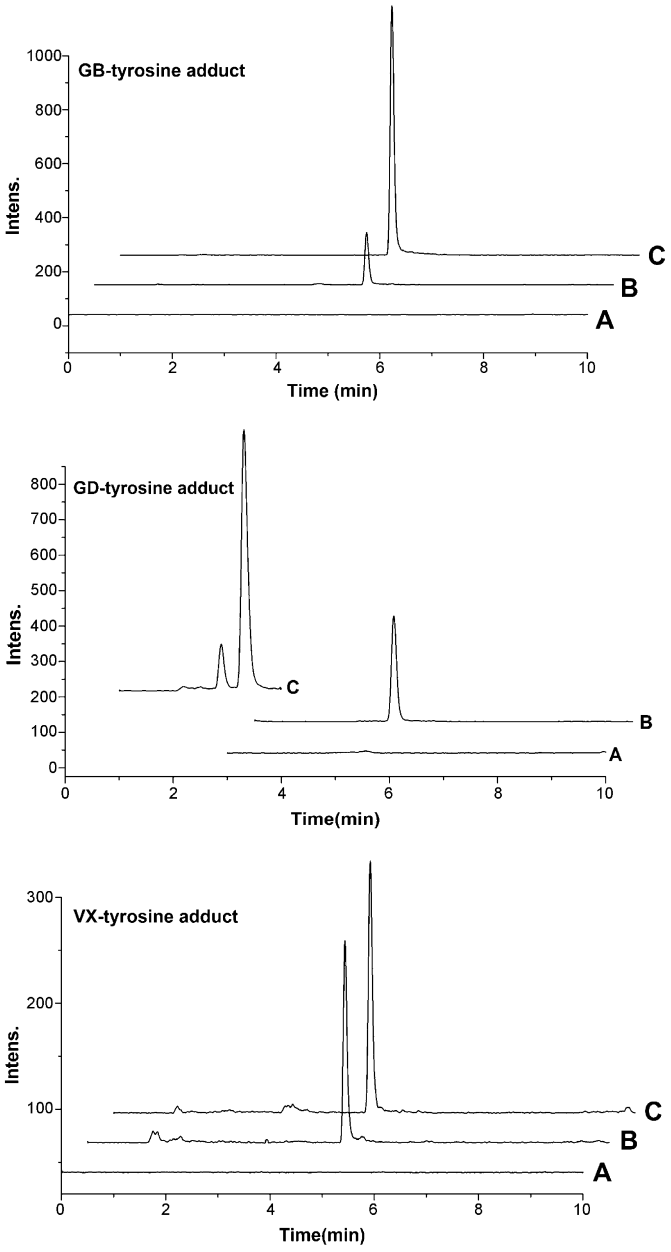

Fig. 3. Extract ion chromatograms of nerve agents adducts with tyrosine and their internal standards in albumin-pronase digestion matrix: (A) blank matrix, (B) synthetic nerve agent-tyrosine adducts: sarin (m/z 302 → 214, 10 ng/mL), soman (m/z 260 → 214, 10 ng/mL) and VX (m/z 288 → 242, 2 ng/mL) and (C) internal standards: D7-sarin (m/z 309 → 215, 60 ng/mL), P-tyrosine (m/z 262 → 216, 50 ng/mL) and D5-VX (m/z 293 → 247, 2 ng/mL).

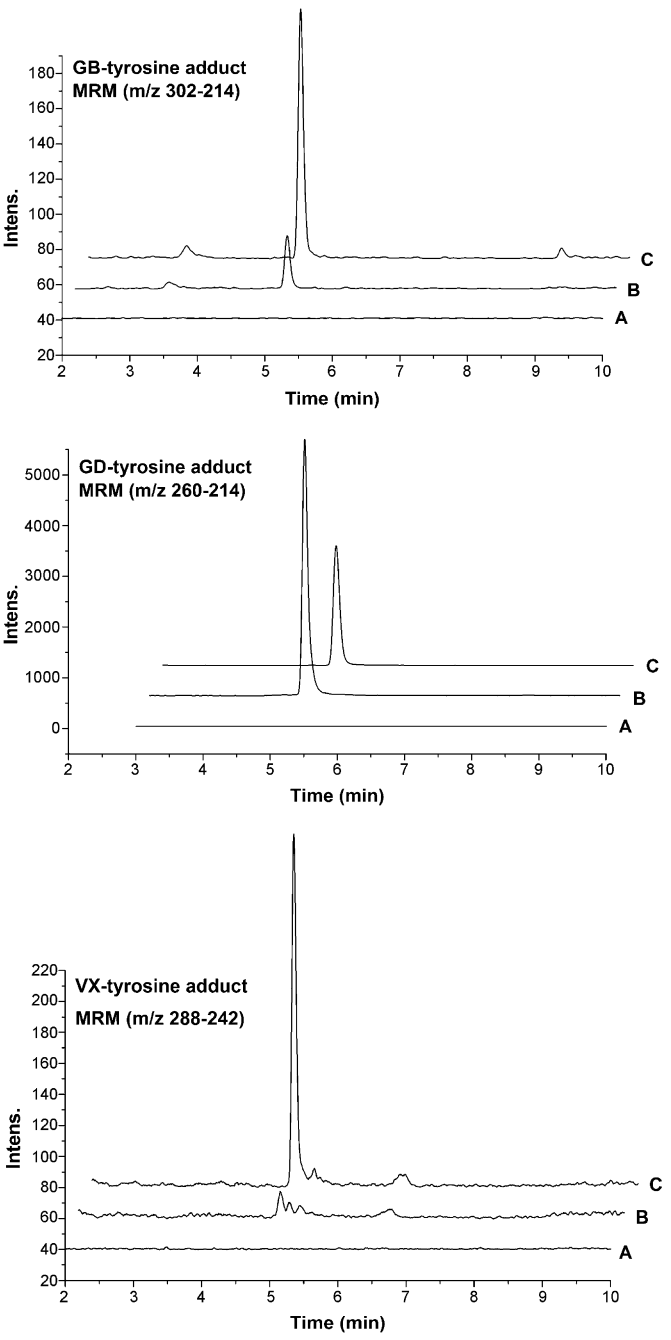

Fig. 4. Representative chromatograms of nerve agents adducts with tyrosine: (A) blank rat plasma, (B) rat plasma exposed to sarin (36 μg/kg), soman (96 μg/kg) and VX (8 μg/kg) and (C) synthetic nerve agent-tyrosine adducts as standards (5 ng/mL for GB-tyrosine, 50 ng/mL for GD-tyrosine and 4 ng/mL for VX-tyrosine, respectively).

was isolated efficiently from rat plasma samples exposed to nerve agents by the use of the HiTrap™ Blue column. The LOD of the method was improved, and interference from other proteins was greatly reduced with this new protocol.

In our research, the C_{18} sorbent was proved to be the most efficient sorbent when compared to other extraction materials for the enrichment of trace soman, sarin and VX adducts in the albumin-pronase digestion solution. To improve the extraction efficiency, we packed SPE cartridges by ourselves using C_{18} sorbent and polypropylene tubes with male luer outlets that have a smaller inner diameter than the commercial cartridges. The retention of the three adducts in C_{18} sorbent was different according to the size of the various alkyl groups of the adducts. The smaller the alkyl group is, the more easily it will be eluted from the sorbent.

3.3. LC–IDMS/MS analysis

Utilisation of LC–IDMS/MS for the quantification of organophosphylated tyrosine adducts demonstrated protocol sensitivity, selectivity and robustness. The mass spectrometer was operated in multiple-reaction monitoring mode (MRM). The transition that had the highest abundance was selected as the quantification transition, while the most specific transitions were defined as qualification

Table 3
The MRM transitions of three adducts and their internal standards.

Compounds	Fragmentor (V)	Quantification		Qualification			
		Transitions	Collision energy (V)	Transitions	Collision energy (V)	Transitions	Collision energy (V)
Sarin-tyrosine adduct	80	302 → 214	15	302 → 260	5	302 → 91	50
D7-s arin-tyrosine adduct	80	309 → 215	15	309 → 261	5	309 → 91	50
Soman-tyrosine adduct	130	260 → 214	10	260 → 118	25	260 → 91	45
P-tyrosine	90	262 → 216	10	262 → 118	20	262 → 91	40
VX-tyrosine adduct	100	288 → 242	10	288 → 214	15	288 → 91	50
D5-VX-tyrosine adduct	100	293 → 247	10	293 → 215	15	293 → 91	50

Table 4
Sensitivity and Linearity of three adducts.

Tyrosine adduct	t_R (min)	Reproducibility of peak area (RSD, %)	Concentration of internal standards (ng/mL)	Regression equation	R^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Sarin-tyrosine adduct	5.2 ± 0.02	5.5	60	$y = 258.7x + 4.973$	0.9987	0.1–1000	0.01	0.05
Soman-tyrosine adduct	5.6 ± 0.01	4.9	50	$y = 191.5x + 416.9$	0.9976	0.5–1000	0.01	0.05
VX-tyrosine adduct	4.9 ± 0.02	7.3	9.2	$y = 7.622x + 3.440$	0.9943	0.1–160	0.05	0.1

transitions. The specific operating conditions for individual compounds were optimised and are listed in Table 3. Analysed by the optimised LC–IDMS/MS methods, the chromatograms of synthetic nerve agent-tyrosine adducts and their deuterated internal standards are shown in Fig. 3.

3.4. Method validation

The specificity of the method was investigated by the analysis of control rat plasma samples. LC–IDMS/MS chromatograms of the rat plasma samples from blank rats, exposed rats and those spiked with nerve agent-tyrosine adducts were visually examined and compared for chromatographic integrity and potential interferences. Representative chromatograms are shown in Fig. 4, which displays the lack of interfering peaks.

The linearity of each curve was determined by plotting the peak area ratio of the adduct to the internal standard *versus* concentration, with both axes expressed as $1/x$. As shown in Table 4, all calibration curves showed good linearity.

Sensitive analytical methods were developed for the detection of sarin, soman and VX tyrosine adducts in matrix-spiked samples. The LOD and LOQ for each compound are also shown in Table 4.

The precision of the method was determined by six replicate analyses of quality control samples in albumin-pronase digestion solution at three concentration levels. The intra-day and inter-day precision values are presented in Table 5.

The results of recovery showed that it was efficiency to extract the adducts utilising the SPE method optimised. The recovery results are also shown in Table 5.

3.5. The analysis of plasma samples from rats exposed to nerve agents

3.5.1. Nerve agent administration and sample collection

Three dose levels of sarin and soman were administered to verify the relationship between the agent dose and the concentration of adducts. Because of reports that the abundance of VX adduct was much lower than that of sarin and soman adducts and the difficulty of detection except at higher concentration levels [14], only a group of rats were exposed with VX in a moderate dose to verify the dose of exposure. All rats of the soman group at the high-dose level (GD-H) had the obvious toxic symptom of nerve agents 1 h after exposure, therefore the blood sample was collected at 1.5 h post-agent injection.

3.5.2. BuChE activity assay

The inhibition ratio of BuChE activity was different with various nerve agents and various exposure doses. The relationship between the exposure dose and the inhibition ratio of BuChE activity is shown in Table 6.

3.5.3. Quantitative detection of the adducts in a rat that was exposed to nerve agents

It has been demonstrated that the isotopic dilution MS method could be used to detect 50 femtogram amounts of agent adducts on-column, despite the complexity of the plasma matrix. The concentration of nerve agent-tyrosine adducts in the exposed rat plasma samples increased obviously with increasing dosage, as shown in Table 6.

It has been reported that toxic symptoms in an animal following an exposure to sarin typically appear at approximately 85% BuChE

Table 5
Intra-day and inter-day precisions, recoveries of three adducts.

Compounds	Concentration (ng/mL)	Precision (RSD, %)		Recovery (%) ($n = 6$)	
		Intra-day ($n = 6$)	Inter-day ($n = 6$)	Average	RSD, %
Sarin-tyrosine adduct	1	4.7	3.2	87	2.7
	10	2.2	2.7	92	3.6
	100	2.4	2.3	96	6.2
Soman-tyrosine adduct	1	5.2	8.3	111	3.1
	10	3.7	13	99	5.7
	100	3.0	9.9	104	3.4
VX-tyrosine adduct	1	3.0	7.4	89	7.3
	10	6.2	3.8	93	5.0
	100	4.7	5.9	98	5.7

Table 6
Inhibition ratio of BuChE activity and concentration of adducts in a rat that was exposed to nerve agents with different dosage ($n = 5$).

Groups	Dose of exposure (μg/kg) ($n = 5$)	Time after exposure (h) ($n = 5$)	Inhibition of BuChE activity (%) ($n = 5$)	Concentration of adduct (ng/mL) ($n = 5$)
Blank	0 (saline of same volume)	48	0	0
GD-L	24	48	12.7 ± 2.0	11.54 ± 2.2
GD-M	48	48	24.8 ± 2.9	83.61 ± 9.6
GD-H	96	1.5	53.2 ± 4.2	136.41 ± 19.5
GB-L	9	48	5.2 ± 1.3	0.19 ± 0.04
GB-M	18	48	12.7 ± 3.1	0.31 ± 0.11
GB-H	36	48	21.8 ± 3.9	0.62 ± 0.19
VX	8	48	43.7 ± 5.9	0.14 ± 0.04

inhibition [19]. The nerve agent-tyrosine adducts were detected at 10–20% plasma BuChE inhibition for soman (1 ng agent/mL plasma) and ≥70% BuChE inhibition for sarin (10 ng agent/mL plasma) in *in vitro* incubations [14]. In our *in vivo* experiment, the adducts can be detected at approximately ≥5% BuChE inhibition for sarin, ≥13% for soman and ≥44% for VX, demonstrating that this method is more sensitive and selective than those that have been previously reported [14]. The results demonstrated that nerve agent adducts with the tyrosine residue of albumin are viable biomarkers following exposure to sub-lethal doses of soman, sarin and VX.

4. Conclusions

A highly sensitive and selective quantification method of liquid chromatography–isotope dilution tandem mass spectrometry was established for nerve agent adducts with albumin in rat post-exposure. Three *O*-(*O*-alkyl methylphosphonyl) tyrosine adducts and their deuterated products were synthesised. The HiTrap™ Blue column was favourable for achieving higher recovery efficiency, lower interference from other proteins and lower detectable concentrations in combination with an optimised SPE protocol. The concentrations of agent adducts in rat plasma samples at different exposure dose levels were determined to show the dose–effect relationships. The developed method could be used to monitor nerve agent exposure and to gain a more comprehensive understanding of the *in vivo* metabolism of nerve agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.032.

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