Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 838 (2006) 21–30
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Detection of human butyrylcholinesterase-nerve gas adducts by liquid chromatography–mass spectrometric analysis after *in gel* chymotryptic digestion☆

Kouichiro Tsuge*, Yasuo Seto

National Research Institute of Police Science, 6-3-1 Kashiwanoha, Kashiwa, Chiba 277-0882, Japan

Received 29 September 2005; accepted 25 February 2006
Available online 29 March 2006

Abstract

To verify the exposure to nerve gas, a method for detecting human butyrylcholinesterase (BuChE)-nerve gas adduct was developed using LC–electrospray mass spectrometry (ESI-MS). Purified human serum BuChE was incubated with sarin, soman or VX, and the adduct was purified by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and digested *in gel* by treatment with chymotrypsin. The resulting peptide mixture was subjected to LC–ESI-MS. From the chymotryptic digest of untreated human BuChE, one peak corresponding to the peptide fragment containing the active center serine residue was detected on the extracted ion chromatogram at *m/z* 948.5, and the sequence was ascertained to be "GESAGAASVSL" by MS/MS analysis. From the chymotryptic digest of the human BuChE-sarin adduct, a singly charged peptide peak was detected on the extracted ion chromatogram at *m/z* 1069.5, and the sequence was ascertained to be "GEXAGAASVSL" by MS/MS analysis (X denotes isopropylmethylphosphonylated serine). The difference in molecular weight (120.0 Da) between the active center peptide fragments corresponding to the untreated BuChE and BuChE-sarin adduct was assumed to be derived from the addition of an isopropyl methylphosphonyl moiety to the serine residue. The formation of human BuChE adducts with soman, VX and an aged soman adduct was confirmed by detecting the respective active center peptide fragments using LC–ESI-MS. To apply the established method to an actual biological sample, human serum was incubated with VX, and the adduct was purified by procainamide affinity chromatography followed by SDS-PAGE. After chymotryptic *in gel* digestion, the ethylphosphonylated active center peptide fragment could be detected, and the structure of the residue was ascertained by LC–ESI-MS analysis.

Keywords: Nerve gas; Butyrylcholinesterase; Adduct; LC–MS; Chymotryptic digestion

1. Introduction

Nerve gases were developed as chemical warfare agents (CWA) during and prior to World War II, and large amounts of the CWA continue to exist and are in storage. In 1994 and in 1995, sarin terrorism cases occurred in Matsumoto city and the Tokyo subway system, which were caused by members of the Japanese cult AUM SHINRIKYO [1,2]. On 2001, 9.11 terrorism involving a US airplane and an anthrax postal mail incident occurred. As a result, it has become clear that, chemical and biological warfare agents constitute an emerging terrorist threat. In forensic cases, it is necessary to prove exposure to CWA. The detection and identification of CWA using GC and GC–MS have been mainly performed concerning site inspections [3].

Nerve gases readily hydrolyze to produce alkyl methylphosphonic acids (RMPA) [4], and it is difficult to detect nerve gases themselves especially in biological samples obtained from victims. Therefore, the detection and identification of the degradation products is a more realistic choice for verifying exposure to a nerve gas. RMPA and its hydrolysis product methylposphonic acid (MPA) are water-soluble, non-volatile compounds. As a result, derivatization is indispensable for GC and GC–MS analysis. Our laboratory routinely uses *tert*-butyldimethylsilylation to derivatize RMPA and MPA, and employs this method for the analysis of environmental [5,6] and biological [7] samples. However, it is difficult to detect this degradation product in a

☆ This paper was presented at the 30th Annual Meeting of the Japanese Society for Biomedical Mass Spectrometry, Osaka, Japan, 8–9 September 2005.

* Corresponding author. Tel.: +81 471 35 8001; fax: +81 471 33 9173.
E-mail address: tuge@nrips.go.jp (K. Tsuge).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.02.054

sample from a victim, because the blood half lives of RMPA and MPA are not very long, and most nerve gases are bound to cholinesterases (ChEs) in the body. In fact, we were not able to detect a sarin hydrolysis product from all of the victims in the Tokyo subway sarin attack [2].

Acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.8), are serine esterases in which the active center contains a serine residue and related charge relay systems [8]. Organophosphorous anticholinesterase agents irreversibly inhibit serine esterase activity, via the phosphorylation of the serine residue at the active center [9]. Therefore, the detection of inhibited ChE, a ChE adduct, seems ideal candidate for verifying nerve gas exposure, because of long-term presence of adduct proteins in a victim's blood. Polhuijs et al. [10] detected sarin from blood samples obtained after the Matsumoto sarin gas incident by the regeneration of sarin from isopropylmethylphosphonylated BuChE using excess fluoride ion. Nagao et al. [11] detected isopropylmethylphosphonic acid (IMPA) which was liberated from inhibited erythrocyte acetylcholinesterase in blood samples from victims of the Tokyo subway sarin gas attack, using immuno-affinity chromatographic purification and phosphatase treatment. Barak et al. [12] demonstrated the direct detection of sarin and soman adducts of human recombinant acetylcholinesterase using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis. Noort et al. established the biomonitoring method of CWA exposure by detecting adducts of biomacromolecules [13].

In our previous study [14], the formation of diisopropylfluorophosphate (DFP) inhibited chymotrypsin was confirmed using tryptic digestion in conjunction with an LC–MS/MS technique. Direct confirmation of the covalent binding of anticholinesterase agents to target enzymes by the mass spectrometric determination of enzyme adducts seems crucial in the elucidation of exposure and poisoning by organophosphorous compounds. Fidder et al. [15] reported the analysis of organophosphate-inhibited human BuChE by LC–MS for the confirmation of nerve gas exposure, where human BuChE was purified by affinity chromatography, and the active center nonapeptide was detected as an adduct marker peptide after the pepsin digestion. In this study, we developed digestion LC–MS method, adopting additional pretreatment procedure of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) after the affinity chromatographic BuChE purification from serum sample to raise the purity of the isolated BuChE and enable *in gel* digestion. We adopted the chymotrypsin as the proteolytic enzyme, because α-chymotrypsin is one of generally used proteolytic enzymes in recent proteome studies, and its digestion efficiency is high. We also applied this method to a blood sample.

2. Experimental

2.1. Reagents

α-Chymotrypsin from bovine pancreas was purchased from Merck (Darmstadt, Germany). Sarin, soman and VX were obtained from the TNO Prince Maurits Laboratory (The Netherlands), and was used with the permission of the Ministry of Economy, Trade and Industry. Other chemicals were of analytical reagent grade. All buffers were prepared with distilled water.

2.2. Purification of butyrylcholinesterase from human serum

BuChE was purified by procainamide affinity chromatography, as described by Ralston et al. [16] with minor modifications. Human blood was collected from a healthy volunteer in our institute, with consent. One thousand milliliters of human serum was treated in one batch. In the first step, the serum was acidified by the addition of sulfuric acid to adjust the pH to 3.5 at 20 °C, and the precipitate was removed by the centrifugation (5000 × *g* for 15 min) at 20 °C. After neutralization of the resulting supernatant with ammonia, ammonium sulfate was added to 45% saturation under 4 °C, and the resulting precipitate was removed by centrifugation (8000 × *g* for 15 min) at 4 °C. Additional ammonium sulfate was then added to the solution to 75% saturation under 4 °C, and the precipitate (45–75% saturation fraction) was collected by centrifugation. The precipitate was dissolved in a small volume of distilled water, and dialyzed twice against 50 mM sodium phosphate buffer (pH 8.0). The dialyzate was supplemented with a saturated solution of sodium chloride, to give a final concentration of 0.1 M, and the solution was applied to procainamide-Sepharose 4B gel (ϕ = 20 mm, *L* = 400 mm) that had been equilibrated with buffer A (0.1 M sodium chloride/50 mM sodium phosphate buffer (pH 8.0)). All operations were carried out using an ÄKTA prime chromatography system (Amersham-Pharmacia Biotech, Uppsala, Sweden) at 4 °C. The column was washed with 500 ml of buffer A. Elution was performed at a flow rate of 2 ml/min with a linear gradient from 100% buffer A to 20% buffer A/80% buffer B (20 mM procainamide in 50 mM sodium phosphate buffer (pH 8.0)) over a period of 40 min. The BuChE fraction was concentrated using a Centricon 50 centrifugal filter unit (Millipore, Billerica, MA), and subjected to Sephacryl S-300HR (ϕ = 24 mm, *L* = 600 mm) gel filtration chromatography. Elution was carried out with 20 mM sodium phosphate buffer (pH 8.0) at a rate of 0.5 ml/min. The BuChE fraction was concentrated by means of a Centricon 50 centrifugal filter to give the purified BuChE.

2.3. Measurement of BuChE activity

BuChE activity was measured using Ellman's spectrophotometric method [17] with minor modifications [18]. The enzyme solution was incubated in 5 mM sodium phosphate buffer (pH 8.0) containing 2 mM butyrylthiocholine and 235 μM 5,5′-dithiobis(2-nitro benzoicacid) (DTNB) for 15 min at 25 °C, and the yellow color produced was measured at 405 nm after termination of the enzyme reaction by the addition of eserine (final 0.18 mM). One unit was defined as the amount of BuChE required to hydrolyze 1 μmol of butyrylthiocholine per minute.

2.4. Formation of nerve gas adduct of human BuChE

A 1 μl aliquot of 0.01% (v/v) sarin, soman or VX in acetonitrile was added to 20 μl of purified human BuChE solution (1.1 μg/μl in 20 mM sodium phosphate buffer (pH 7.2) or 20 mM Tris–HCl buffer (pH 8.0)), and the mixture was incubated at 37 °C for 10 min on a block heater. In a control experiment, 1 μl of acetonitrile was added in place of the nerve gases. The complete formation of adduct was confirmed by assaying the BuChE activity of the mixture.

2.5. Sodium dodecylsulfate-polyacrylamide gel electrophoresis

SDS-PAGE was carried out by Laemmli's method [19] using an ATTO AE6530-P mini slab electrophoresis unit (Tokyo, Japan). The final acrylamide concentration was 8% (w/v) for the separating gel and 4% (w/v) for the stacking gel. All samples were mixed with the same volume of 2× Laemmli sample buffer [0.1 M Tris–HCl buffer (pH 6.6), 200 μM dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue, 20% glycerol], and denaturated at 98 °C for 10 min. After loading the samples, electrophoresis was carried out with a constant voltage of 80 V for 3 h. After the electrophoresis, the gel was stained in 0.1% Coomassie Brilliant Blue R250/20% methanol/0.5% acetic acid, and then destained in 30% methanol.

2.6. Chymotryptic in gel digestion

The stained band at around 86 kDa on the SDS-PAGE gel was excised, and chopped into small pieces. The gel pieces were destained by the addition of 300 μl of 60% acetonitrile/40% 20 mM sodium bicarbonate buffer (pH 8.8). This destaining step was repeated until the background blue color completely disappeared. After removing the solution, the gels were dried on a centrifugation evaporator (Sakuma Seisakusho Ltd., Tokyo, Japan) without heating. A 5 μl of 0.2% (v/v) Tween-20 containing 20 mM sodium bicarbonate buffer (pH 8.0) was added to swell the gel. A 2 μl volume of chymotrypsin solution (10 μg/μl) was added to the above solution, and the mixture was incubated at 37 °C for 30 min. The gel was filled with 200 μl of sodium bicarbonate buffer (pH 8.0) and incubated at 37 °C for 18 h. The digestion was stopped by the addition of 5 μl of formic acid.

2.7. Liquid chromatography–mass spectrometry

A Q-TOF2 mass spectrometer (Micromass, Altrincham, UK), equipped with a nebulized electrospray ionization (ESI) z-spray source was used in the experiments. The mass spectrometer was coupled with Agilent 1150 HPLC systems (Palo Alto, CA, USA). A μs-CrestPak C18S column (JASCO, Tokyo, Japan, 2.2 mm × 150 mm) was used as separation column. The stationary phase was equilibrated with 95% solution A/5% solution B (solution A, 0.22% formic acid in water, solution B, 0.2% formic acid/99.8% acetonitrile). Elution was performed at a flow rate of 0.2 ml/min with a linear gradient from 95% solution A/5% solution B to 50% solution A/50% solution B over a period of 50 min with a 5 min hold at the initial conditions. All of the column eluate was introduced into the electrospray interface. The ionization was carried out in positive ionization mode. The capillary voltage, cone voltage and microchannel plate (MCP) detector voltage were set at 3000, 35 and 2200 V, respectively. The collision energy was set at 10 eV. Desolvation was carried out by blowing nitrogen gas at 250 °C. Instrumental operation, data acquisition and data analysis were performed using the MassLynx 3.2 software (Micromass), on a Windows NT workstation. In the MS/MS measurement, the mass spectrometric parameters were adjusted as follows: the MCP voltage was set at 2500 V, and aperture2 was 18 V. Argon gas was used as the collision gas, and the collision energy was optimized from 20 to 45 V.

An Agilent LC-MSD Trap mass spectrometer equipped with an ESI ion source was also used. The mass spectrometer was coupled with an Agilent 1100 series HPLC equipped with a micro flow pump system. A Zorbax SB C18 300A column (Agilent, 0.5 mm × 150 mm) was used as the separation column. The stationary phase was equilibrated with 95% solution A/5% solution B. Elution was performed at a flow rate of 20 μl/min with a linear gradient from 95% solution A/5% solution B to 50% solution A/50% solution B over 50 min with a 5 min hold at the initial conditions. All of the column eluate was introduced into the electrospray interface. Desolvation was carried out by blowing nitrogen gas at 300 °C. Instrumental operation, data acquisition and data analysis were performed using the ChemStation software (Agilent), on a Windows XP workstation.

2.8. Pretreatment of a serum sample treated with nerve gas

A 5 ml aliquot of serum was treated with 50 μl of 0.01% VX in acetonitrile, and incubated at 37 °C for 10 min. The serum was diluted with 9 volumes of buffer C (50 mM sodium chloride/20 mM sodium phosphate buffer (pH 8.0)), and loaded on a procainamide affinity gel ($\phi = 10$ mm, $L = 140$ mm) at a flow rate of 2.0 ml/min, operated by an ÄKTA prime chromatography system. The column was washed with 150 ml of buffer C at a flow rate of 2 ml/min, and the elution was performed at a flow rate of 1 ml/min with a linear gradient from 100% buffer C to buffer C containing 16 mM procainamide over a period of 40 min. The BuChE fraction was concentrated by Centricon 50 centrifugal filter unit, and applied to SDS-PAGE followed by chymotryptic *in gel* digestion and LC–MS analysis.

2.9. Safety consideration

Sarin, soman and VX are highly toxic upon inhalation and exposure to the skin. These compounds must be handled with special care using protective gear within a fume food.

3. Results and discussion

3.1. Purification of BuChE from human serum

BuChE was isolated and purified from 1000 mL of pooled human serum as shown in Section 2.2. Table 1 indicates the

Table 1
Purification stage for BuChE from human serum

	Sample volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (fold)
Serum	1000	520	66000	0.008	1
Acid precipitation/$(NH_4)_2SO_4$ precipitation (50–75% saturation)	240	40	2500	0.16	20
Procainamide affinity chromatography	1.7	180	5.9	31	3800
Sephadex G-200 gel filtration	48	140	1.3	110	14000
Ultrafiltration (concentration)	0.75	85	0.69	120	15000

purification stages. A 0.69 mg of human BuChE was purified, and purification factor was about 15,000-fold. The protein ran as a single band on SDS-PAGE (Fig. 1) and had a molecular weight of approximately 86 kDa.

3.2. LC–MS analysis of untreated human BuChE

The amino acid sequence of human serum BuChE was elucidated using the Edman degradation method [20]. Identification of the BuChE gene was also carried out [21]. The primary amino acid sequence of human BuChE is shown in Fig. 2, which includes a 28-mer of a signal peptide. Serine 226 residue is the active center residue. Nerve gases react with the BuChE active center via covalent binding with the 226th serine residue, leading to the formation of a phosphonate ester. Human serum BuChE is a tetramer of 86 kDa subunits [22], and it is difficult to discriminate the adduct molecule from an unmodified one by mass spectrometric methods, because of the low mass spectrometric resolution and multiple molecular peaks due to multiple monoisotopic peaks and sugar chain heterogeneity. Instead, we targeted a proteolytic digest of the protein, for which the molecular weights were within the scope of ESI-MS measurements. We initially examined tryptic digestion, but unfortunately the peptide containing active center serine residue had a molecular weight of 2928 Da, and so it was difficult to analyze such a high molecular weight peptide. Fidder et al. [15] also adopted pepsin digestion instead of tryptic digestion for the LC–MS analysis of BuChE adducts. We adopted chymotryptic digestion, which provided an analyzable active center peptide from the putative proteolytic digestion pattern (Fig. 2). SDS-PAGE pretreatment was adopted to isolate BuChE from the remaining unreacted nerve gases, because the frequently used ultrafiltration pretreatment did not provide a reasonable recovery of the adduct (data not shown), which is probably due to adsorption of the adduct to the filter surface. After the separation of the adduct by SDS-PAGE, the target protein was digested *in gel*, a procedure that permitted both effective digestion and reasonable recovery.

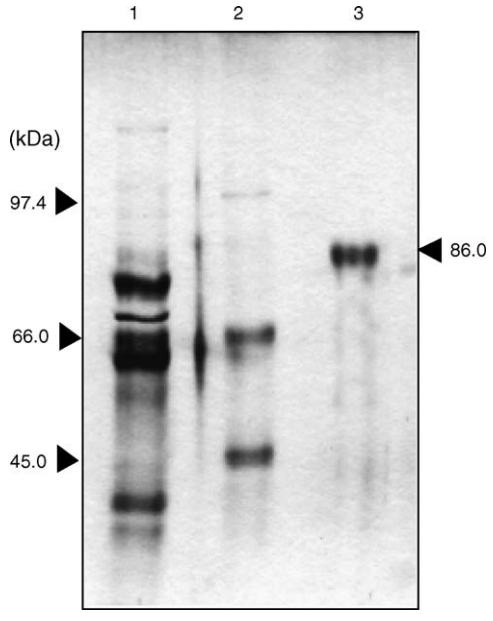

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of human serum ammonium sulfate precipitation fractionation and purified butyrylcholinesterase. Lane 1: 40–75% saturated ammonium sulfate precipitate of serum. Lane 2: molecular weight marker proteins (phosphorylase b: 97.4 kDa, bovine serum albumin: 64.0 kDa, ovalbumin: 45.0 kDa). Lane 3: 2 μg of purified human BuChE. 8% polyacrylamide gel, Coomassie Brilliant Blue R250 stain.

Fig. 3A shows the total ion chromatogram of blank sample which was prepared by the blank running on the SDS-PAGE and *in gel* digesting by the chymotrypsin. Some peaks eluting between 15 and 30 min were observed, which were presumably ascribed to the peptide derived from self-digested α-chymotrypsin. Fig. 3B–F shows the extracted ion chromatograms of *m/z* 948.5, 1068.5, 1054.5, 1110.5 and 1026.5, respectively. Minor peaks were observed on the extracted ion chromatograms of *m/z* 1054.5 and 1110.5, but these were not related to the adduct peptide, and also did not interfere with the adduct peptide detection.

As shown in Fig. 4A, many peptides provided by chymotryptic digestion were obtained on the total ion chromatogram. Fragmentation information after chymotryptic digestion was also obtained using the "MS Digest" software (UCSF Mass Spectrometry Facility, USA) [23]. The sequence of the peptide containing active center serine residue was ascertained to be "GESAGAASVSL" (molecular mass: 948.46). The third serine residue is the active center serine residue. Fig. 4B shows the extracted ion chromatogram of *m/z* 948.5 which corresponds to the singly protonated active center peptide fragment. In the mass spectrum of one peak at 19.4 min as arrowed, a singly protonated molecular ion (*m/z* 948.5) was observed (Fig. 5A). The other peaks were not related to the target peptide. Fig. 5B shows the product ion spectrum of *m/z* 948.5 ion as the precursor ion. The b-series fragment ions from b_6 to b_{10} and the corresponding dehydrated ions were observed. However, other fragment ions (b_1–b_5, y-series and other fragment) were not observed. The sequence of this peptide fragments were ascertained to be

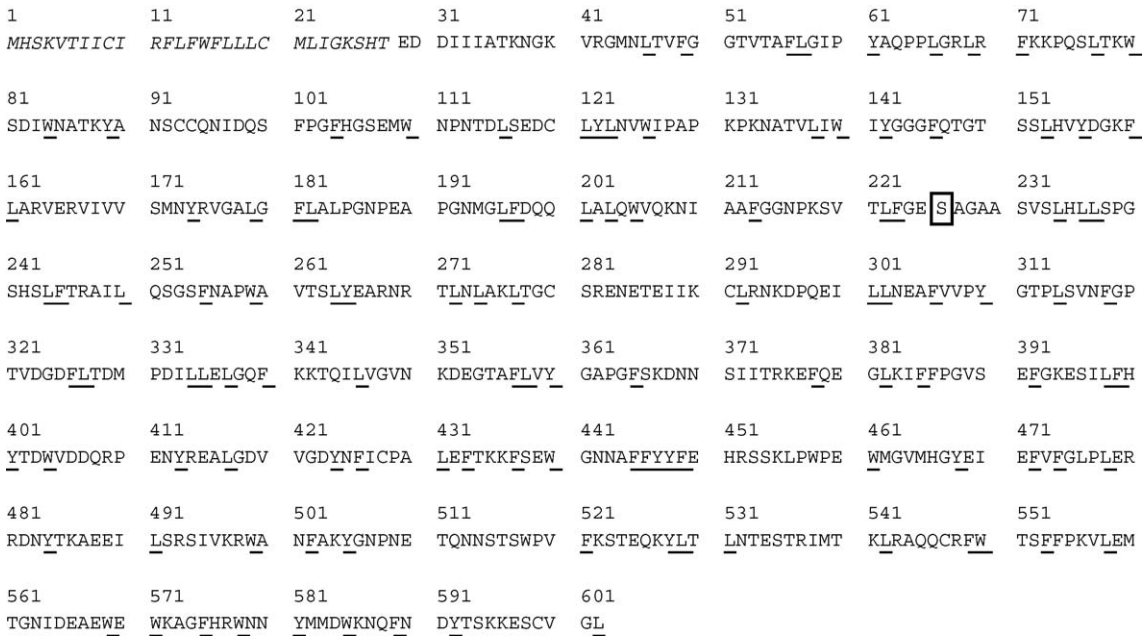

Fig. 2. Primary amino acid sequence of human BuChE. Underlined residues indicate positions which would be expected to be cleaved by chymotrypsin. This sequence contains a 28 mer signal peptide (italic) at N-terminal. The square enclosing the 226th residue indicates the active center serine residue.

"GESAGAASVSL". On the extracted ion chromatogram of *m/z* 1068.5, 1054.5, 1110.5 or 1026.5, neither peak was detected at 22.3 min (sarin adduct peptide), 20.1 min (VX adduct peptide), 26.2 min (soman adduct peptide) nor 21.9 min (aged soman adduct peptide), respectively (data not shown).

3.3. LC–MS analysis of BuChE-sarin adduct

BuChE was reacted with sarin in phosphate buffer (pH 7.2), and the adduct was subjected to SDS-PAGE and chymotryptic *in gel* digestion. On the extracted ion chromatogram of *m/z* 1068.5 corresponding to the singly protonated molecular ion of the active center peptide derived from BuChE-sarin adduct, one peak was obtained, with a retention time of 22.3 min as arrowed (Fig. 6A). The other peaks were not related to the target peptide. On the extracted ion chromatogram of *m/z* 948.5, the 19.4 min peak (corresponding to the untreated active center peptide) was not observed (data not shown). In the mass spectrum of the 22.3 min, peak of the BuChE-sarin adduct, a singly protonated molecular ion (*m/z* 1068.5) was observed (data not shown). Fig. 7A shows the product ion spectrum of *m/z* 1068.5 peak ion as precursor ion. The fragment ions of *m/z* 930.5, 799.5, 712.5, 613.4, 526.3 and 455.2 were observed, which were identical to the fragment ions of the [b-H_2O] series of the active center peptide of untreated BuChE (Fig. 4B). These ions can be attributed to b-series ions in which the isopropylmethylphosphonic acid moiety had been eliminated from the adduct peptide.

3.4. LC–MS analysis of VX-inhibited human BuChE

BuChE was reacted with VX in phosphate buffer (pH 7.2), and the isolated adduct subjected to SDS-PAGE and chymotryptic *in gel* digestion. As shown in Fig. 6B, on the extracted chromatograms of *m/z* 1054.5, corresponding to the singly protonated molecular ion of active center peptide derived from BuChE-VX adduct, one major peak was obtained with a retention time of 20.1 min as arrowed. On the extracted ion chromatogram of *m/z* 948.5 corresponding to the unmodified active center peptide, the 19.4 min peak was not observed (data not shown). In the mass spectrum of the 20.1 min peak of the BuChE-VX adduct, a singly protonated molecular ion (*m/z* 1054.5) was observed (data not shown). Fig. 7B shows the product ion spectrum of the *m/z* 1054.5 peak ion as the precursor ion. Fragment ions of *m/z* 930.5, 799.4, 712.4, 613.4, 526.3 and 455.2 were observed, which were identical with the fragmentation ions of the [b-H_2O] series obtained for the BuChE-sarin adduct (Fig. 7A).

We determined whether or not heat denaturated BuChE was phosphonylated. Purified BuChE was incubated at 95 °C for 10 min, which leads to complete inactivation of the enzyme. The heat-treated enzyme was reacted with VX, and the adduct subjected to SDS-PAGE and chymotryptic digestion. On the extracted ion chromatogram at *m/z* 948.5, 19.4 min peak was obtained. Instead, on the extracted ion chromatogram at *m/z* 1054.5, the 20.1 min peak was not observed (data not shown). Therefore, the intact enzyme structure is required for phosphonylation by the nerve gas.

3.5. LC–MS analysis of soman-inhibited human BuChE

The ChE-nerve gas adduct is known to undergo aging, and as a result, two types of adducts can be anticipated in which the active center serine is attached to either an alkylmethylphosphonyl moiety or a methylphosphonyl moiety. Aging is the result of enzymatic hydrolysis and elimination of the alkyl function from the active center phosphonyl residue. Within nerve gases,

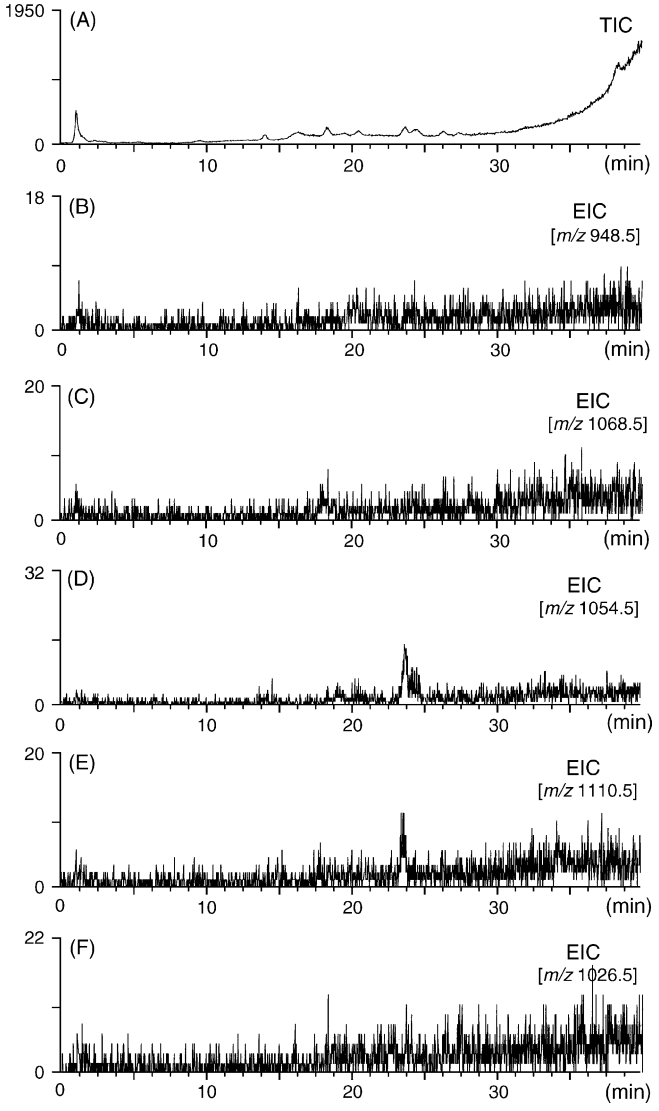

Fig. 3. Total ion and extracted ion chromatograms of blank sample that was incubated with chymotrypsin analyzed by Q-TOF2. (A) Total ion chromatogram. Extracted ion chromatogram at *m/z* (B) 948.5, (C) 1068.5, (D) 1054.5, (E) 1110.5 and (F) 1026.5.

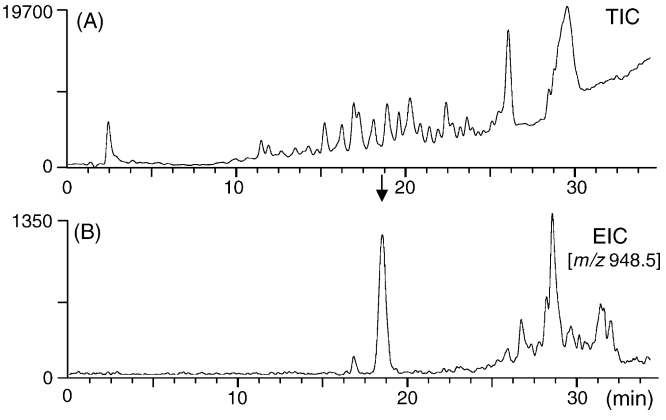

Fig. 4. Total ion and extracted ion chromatograms of chymotryptic digest of untreated human BuChE analyzed by Q-TOF2. (A) Total ion chromatogram and (B) extracted ion chromatogram at *m/z* 948.5. Arrow indicates the peak of the active center peptide.

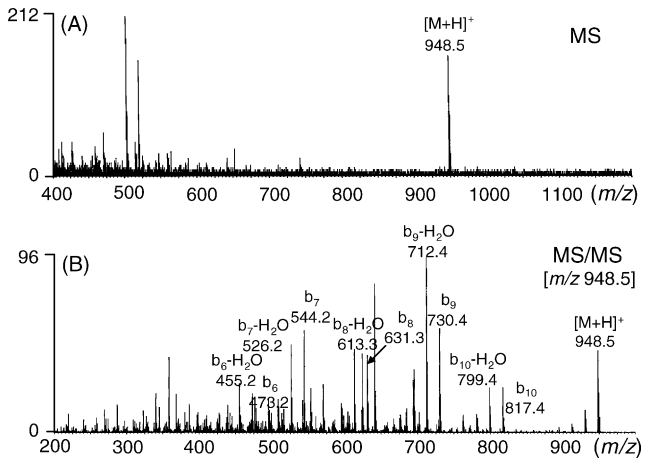

Fig. 5. Mass spectra of the peptide fragment (19.4 min, Fig. 4) containing active center serine residue obtained from untreated human BuChE. (A) Mass spectrum and (B) product ion spectrum of the precursor ion *m/z* 948.5.

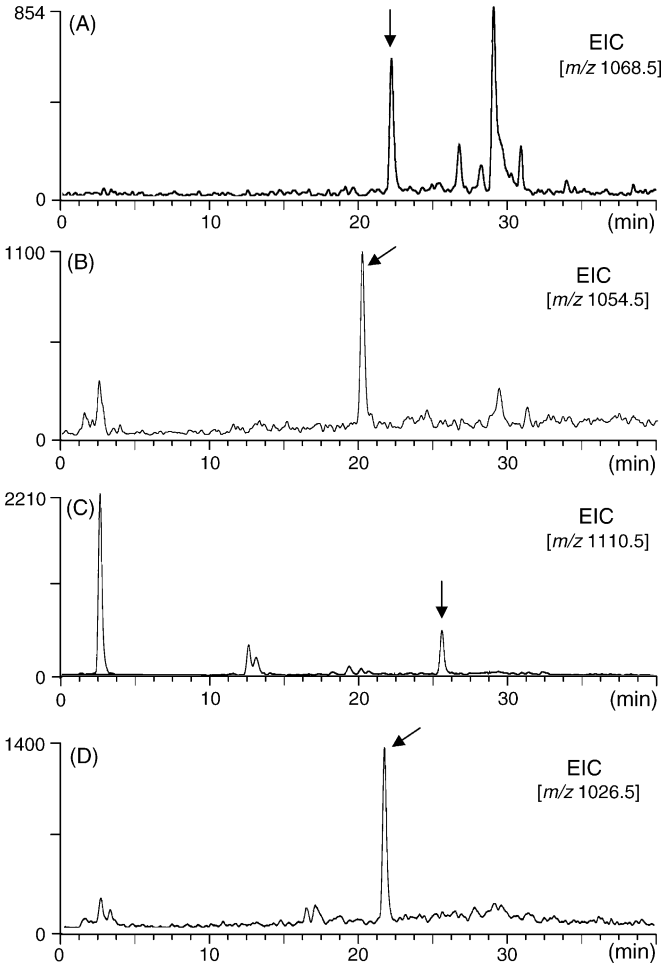

Fig. 6. Total ion and extracted ion chromatograms of a chymotryptic digest of sarin-inhibited (A), VX inhibited (B), soman-inhibited (C) and aging soman-inhibited (D) human BuChE analyzed by Q-TOF2. Arrow indicates the peak of the active center peptide.

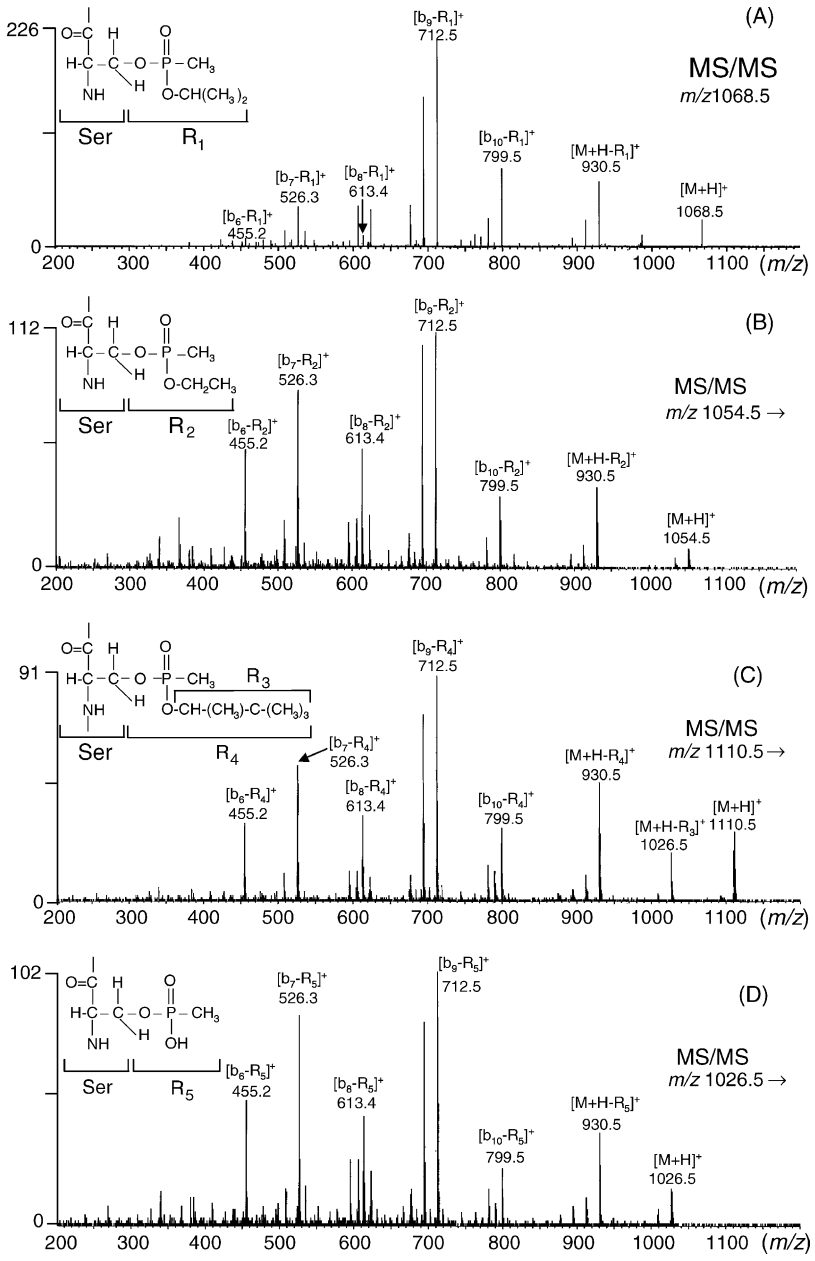

Fig. 7. Product ion spectra of the peptide fragments containing the active center serine analyzed by Q-TOF2. (A) Sarin adduct (precursor ion: *m/z* 1068.5), (B) VX adduct (precursor ion: *m/z* 1054.), (C) soman adduct (precursor ion: *m/z* 1110.5) and (D) soman adduct, aged enzyme (precursor ion: *m/z* 1026.5).

aging of the soman adduct proceeds at the highest rate. In the case of the rat AChE-soman adduct, its half-life is estimated to be about 10 min [24]. BuChE was reacted with soman in phosphate buffer (pH 7.2), and the adduct subjected to SDS-PAGE and chymotryptic digestion. On the extracted ion chromatograms of *m/z* 1110.5, a peak with a retention time of 26.2 min was observed as arrowed (Fig. 6C), corresponding to the active center peptide containing a pinacolylmethylphosphonyl moiety. Also as shown in Fig. 6D, on the extracted ion chromatograms of *m/z* 1026.5, a peak with a retention time of 21.9 min was observed as arrowed, corresponding to the active center peptide being combined with a methylphosphonyl moiety. Singly protonated molecular ions (*m/z* 1110.5 and 1026.5) were observed, respectively (data not shown). Fig. 7C shows the product ion spectrum of the 26.2 min peak as the precursor ion of *m/z* 1110.5. Fig 7D shows the product ion spectrum of the 21.9 min peak as the precursor ion *m/z* 1026.5. Fragment ions at *m/z* 930.5, 799.4, 712.4, 613.3, 526.2 and 455.2 were observed in both the product ion spectra, which can be attributed to the elimination of a pinacolylmethylphosphonic acid (or a methylphosphonic acid) from the adduct peptide. The collision-induced fragmentation of the modified active center serine peptide appears to proceed by the same mechanism. The first step is the elimination of the pinacolyl function from the molecular ion of the pinacolylmethylphosphonylated active center peptide, resulting in the formation of the fragment ion ($[M + H - R_3]^+$, *m/z* 1026.5).

Elimination of methylphosphonic acid next results in the formation of the fragment ion of *m/z* 930.5 ($[M+H-R_4]^+$). The formation of an alkyl function eliminated fragment ion at *m/z* 1026.5 did not occur in the sarin nor VX adducts. It is probable that the bulky pinacolyl function may be easily released from the pseudomolecular ion during the collision process, compared to the phosphonyl function, which contains small ethyl or isopropyl ester.

Fidder et al. [15] also reported that the active center nonadeca peptide obtained by the pepsin digestion of soman inhibited human serum BuChE showed the same fragmentation pattern as ours, with the elimination of the methylphosphonic acid moiety followed by b-series fragmentation. Instead, the 29-mer active center peptide obtained by tryptic digestion from soman inhibited BuChE exerted y-series and y-H_2O series fragmentation. Our previous paper also indicated that the active center 25-mer peptide obtained from tryptic digests of diisopropylfluorophosphate (DFP) inhibited bovine pancreas α-chymotrypsin exerted y-series and b-series fragmentation without loss of the phosphoryl moiety [14]. In spite of the novel fragment pattern of the product ion spectrum of the active center peptides, the LC–MS method seems appropriate for verifying the occurrence of nerve gas exposure. The peak of the active center undeca-peptide (*m/z* 948.5) disappeared after the addition of all the nerve gases examined, and a characteristic peptide peak appeared, indicating phosphonylation of the active center of BuChE.

The rate of aging is reported to depend on the pH of the solution [25]. We also incubated BuChE with soman in Tris–HCl buffer (pH 8.0), which was anticipated to suppress aging reactions compared with more acidic conditions. On the extracted ion chromatogram at *m/z* 1110.5, the 26.2 min peak area was significantly larger for the case of pH 8.0 than 7.2, and on the extracted ion chromatogram of *m/z* 1026.5, the 21.9 min peak area was significantly smaller for the pH 8.0 conditions than the pH 7.2 conditions. Indeed, at the pH 7.2 conditions, the peak area ratio of *m/z* 1110.5 (soman conjugate) and *m/z* 1026.5 (MPA conjugate) on the extracted ion chromatogram was 0.82. Otherwise, the peak area ratio of *m/z* 1110.5 and 1026.5 was 2.66 at the pH 8.0 conditions (data not shown).

3.6. Detection of the BuChE-VX adduct from human serum

In order to detect BuChE-nerve gas adducts in biological samples with a high sensitivity, we adopted an additional BuChE purification procedure involving procainamide affinity chromatography. We examined the BuChE-VX adduct in order to determine the efficiency of detection. It was impossible to detect low levels of the BuChE-VX adduct using the LC-Q-TOF MS system. Instead, we selected an ion-trap type mass spectrometer (Agilent LC-MSD Trap), with capillary C_{18} column HPLC system for use. The doubly protonated molecular ion at *m/z* 527.8 and singly protonated molecular ion at *m/z* 1054.6 were observed for the active center peptide obtained (Fig. 8B) from the BuChE-VX adduct at a retention time of 25.1 min (Fig. 8A). Fig. 8C shows the product ion spectrum (MS^2) of the adduct peptide, in which the precursor ion was selected as *m/z*

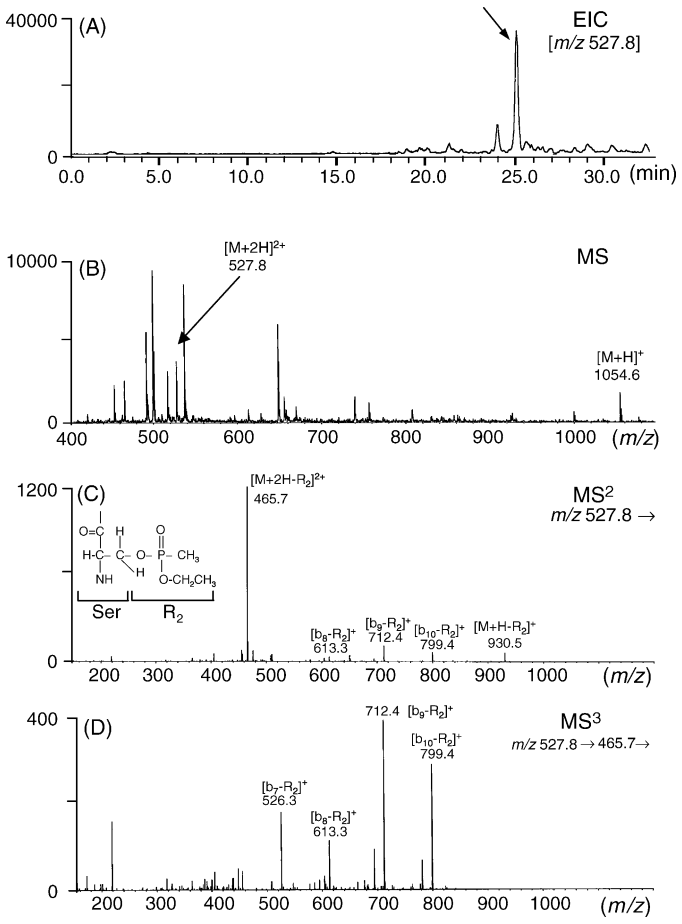

Fig. 8. Extracted ion chromatogram of a chymotryptic digest of VX-inhibited human BuChE and mass spectra of the active center peptide analyzed by LC-MSD Trap. (A) Extracted ion chromatogram at *m/z* 1054.5, (B) mass spectrum of 24.2 min peak in (A), (C) product ion spectrum (MS^2) of the precursor ion *m/z* 527.8 in (B) and (D) product ion spectrum (MS^3) of the precursor ion *m/z* 465.7 in (C).

527.8. A doubly charged fragment ion was observed ion at *m/z* 465.7 as a base peak, which is assigned to the doubly protonated des-ethylmethylphosphonic acid fragment ion. Fragment ions at *m/z* 930.5, 799.3, 712.3 and 613.3 were also observed, which can be attributed to the protonated des-ethylmethylphosphonic acid active center ion and corresponding b-series fragment ions. The following MS^3 measurement was carried out for getting more sequence information. Fig. 8D shows the product ion spectrum (MS^3) of the fragment ion of doubly charged *m/z* 465.7 (Fig. 8C). Des-ethylmethylphosphonic acid b-series fragment ions (b_7–b_{10}) were observed at *m/z* 799.4, 712.3, 613.3 and 526.2. This fragment pattern was similar to the MS/MS spectrum shown in Fig. 7B, obtained using the Q-TOF2 mass spectrometer.

In this experiments, the limit of detection (LOD) of the adduct was estimated not by directly measuring the VX-BuChE adduct molecules but measuring the ethylmethylphosphonylated active center peptide. A 10 μg sample of BuChE was incubated with 50 ng of VX, and subjected to SDS-PAGE. The 86 kDa band was subjected to chymotryptic *in gel* digestion. This

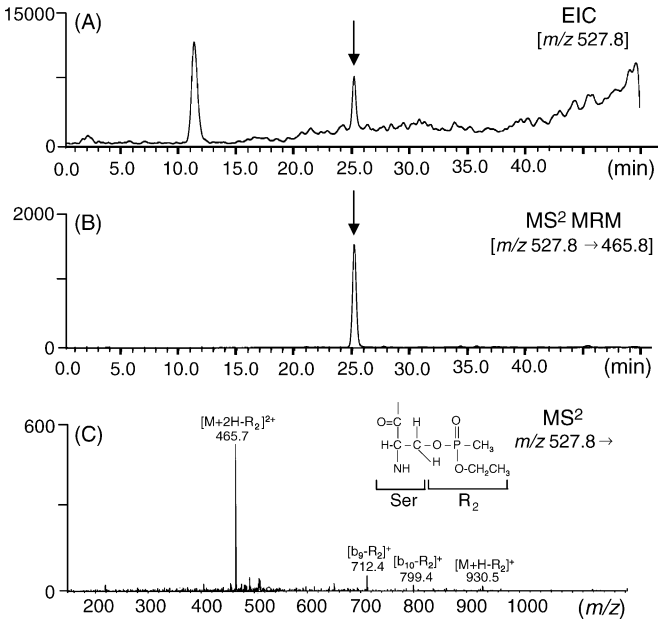

Fig. 9. Liquid chromatography–mass spectrometry of active center peptide of BuChE-VX adduct purified from VX spiked serum analyzed by LC-MSD Trap. (A) Extracted ion chromatogram at *m/z* 1054.5, (B) multiple reaction monitoring chromatogram of *m/z* 527.8–465.7 and (C) product ion spectrum of *m/z* 527.8 ion of 24.2 min peak.

digested sample mixture was used as a standard VX-combined active center peptide. This solution was diluted and analyzed by LC–MS/MS to prepare a standard curve. Multiple reaction monitoring (MRM) was performed using *m/z* 527.8 as a precursor ion and *m/z* 465.7 as an extracted ion, and the 25.1 min peak area on the MRM chromatogram was plotting against the amount of chymotryptic digest of BuChE-VX adduct. A linear standard curve was obtained ranging from 20 to 2500 ng BuChE per injection, and detection limit (S/N = 3) was estimated to be 4 ng BuChE per injection. Assuming that the recovery and digestion efficiency was constant with the amount of treated BuChE, the detection limit was calculated to be 55 pg (53 fmol) of active center peptide per injection. This sensitivity seems satisfactory for the analysis of BuChE-nerve gas adducts obtained from victim's blood, because the amount of BuChE in human serum is assumed to be approximately 80 nM [26].

The BuChE-VX adduct was formed by the addition of 50 μl of 0.01% (v/v) VX acetonitrile solution to 5 ml of control human serum, and the BuChE activity was fully inhibited. The VX treated serum sample was subjected to procainamide affinity chromatography, and chymotryptic *in gel* digestion, followed by LC–MS/MS analysis. Fig. 9A shows the extracted ion chromatogram of *m/z* 527.8 of the chymotryptic digest of the BuChE-VX adduct. One peak was obtained at 25.2 min as arrowed, which was identical to the standard BuChE-VX adduct (Fig. 8A). Fig. 9B shows the MRM chromatogram, in which the precursor and extracted ion were *m/z* 527.8 and 465.7, respectively. The S/N ratio of the target peptide peak as arrowed was improved significantly compared with the extracted ion chromatogram (Fig. 9A). Fig. 9C shows the product ion spectrum (MS^2) of the target peak of *m/z* 527.8. Des-ethylmethylphosphonic acid b-series fragment ions (b_9 and b_{10}) were observed at *m/z* 799.4 and 712.4, in which the mass spectrum pattern was identical with the standard BuChE-VX adduct (Fig. 8C).

To estimate the detection limit for serum sample, we prepared 25% activity inhibited serum sample. A 5.0 ml of serum was mixed with 50 μl of 1 ng/μl VX in acetonitrile. The VX adduct was purified and digested. A part of the chymotryptic digest was subjected to LC–MS. The adduct peptide equivalent to about 1.5 pmol per injection was obtained comparing the above estimated calibration curve, and then the lowest detection level of the BuChE activity inhibition was calculated to be about 1% inhibition.

In conclusion, a BuChE-nerve gas adduct was detected from 5 ml of control human serum inhibited by VX, using the established LC–MS method, in which nerve gas combined with the active center undeca-peptide is used as an identification marker.

Acknowledgment

This research was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Young Scientists (B), 16790368, 2005.

References

[1] White Paper on Police 1994 and 1995, National Police Agency, Government of Japan.
[2] Y. Seto, N. Tsunoda, M. Kataoka, K. Tsuge, T. Nagano, in: A.A. Tu, W. Gaffield (Eds.), Natural and Selected Synthetic Toxins-Biological Implications, American Chemical Society, Washington, DC, 1999, ACS Symposium Series, vol. 745, p. 318.
[3] M. Rautio (Ed.), Recommended Operating Procedures for Sampling and Analysis in the Verification of Chemical Disarmament, The Ministry of Foreign Affair of Finland, Helsinki, 1994.
[4] A. Verweij, H.L. Boter, Science 204 (1979) 616.
[5] M. Kataoka, N. Tsunoda, H. Ohta, K. Tsuge, H. Takesako, Y. Seto, J. Chromatogr. A 824 (1998) 211.
[6] M. Kataoka, K. Tsuge, H. Takesako, T. Hamazaki, Y. Seto, Environ. Sci. Technol. 35 (2001) 1823.
[7] M. Kataoka, Y. Seto, J. Chromatogr. B. 795 (2003) 123.
[8] D.M. Blow, J.J. Birktoft, B.S. Hartley, Nature 221 (1969) 337.
[9] A.R. Thompson, R.I. Ellin, Biochim. Biophys. Acta 220 (1970) 101.
[10] M. Polhuijs, J.P. Langenberg, H.P. Benschop, Toxicol. Appl. Pharmacol. 146 (1997) 156.
[11] M. Nagao, T. Takatori, Y. Matsuda, M. Nakajima, H. Iwase, K. Iwadate, Toxicol. Appl. Pharmacol. 144 (1997) 198.
[12] R. Barak, A. Ordentlich, D. Barak, M. Fischer, H.P. Benschop, L.P.A. de Jong, Y. Segall, B. Velan, A. Shafferman, FEBS Lett. 407 (1997) 347.
[13] D. Noort, H.P. Benschop, R.M. Black, Toxicol. Appl. Pharmacol. 184 (2002) 116.
[14] K. Tsuge, Y. Seto, J. Chromatogr. B 776 (2002) 79.
[15] A. Fidder, A.G. Hulst, D. Noort, R. de Ruiter, M.J. van der Schans, H.P. Benschop, J.P. Langenberg, Chem. Res. Toxicol. 15 (2002) 582.
[16] J.S. Ralston, A.R. Main, B.F. Kilpatrick, A.L. Chasson, Biochem. J. 211 (1983) 243.

[17] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M. Featherstone, Biochem. Pharmacol. 7 (1961) 88.
[18] T. Shinohara, Y. Seto, Reports Nat. Res. Inst. Police Sci. 38 (1985) 178 (in Japanese).
[19] U.K. Laemmli, Nature 227 (1970) 680.
[20] O. Lockridge, C.F. Bartels, T.A. Vaughan, C.K. Wong, S.E. Norton, L.L. Johnson, J. Biol. Chem. 262 (1987) 549.
[21] M. Arpagaus, M. Kott, K.P. Vatsis, C.F. Bartels, B.N. La Du, O. Lockridge, Biochemistry 29 (1990) 124.
[22] H. Muensch, H.W. Goedde, A. Yoshida, Eur. J. Biochem. 70 (1976) 217.
[23] K.R. Clauser, P.R. Baker, A.L. Burlingame, Anal. Chem. 71 (1999) 14.
[24] B.G. Talbot, D.R. Anderson, L.W. Harris, L.W. Yarbrough, W.J. Lennox, Drug. Chem. Toxicol. 11 (1988) 289.
[25] A. Saxena, C. Viragh, D.S. Frazier, I.M. Kovach, D.M. Maxwell, O. Lockridge, B.P. Doctor, Biochemistry 37 (1998) 15086.
[26] D.K. Mayers, Biochem. J. 51 (1952) 303.