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Retrospective Detection of Exposure to Organophosphorus Anti-Cholinesterases: Mass Spectrometric Analysis of Phosphylated Human Butyrylcholinesterase

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In this paper a novel and general procedure is presented for detection of organophosphate-inhibited human butyrylcholinesterase (HuBuChE), which is based on electrospray tandem mass spectrometric analysis of phosphylated nonapeptides obtained after pepsin digestion of the enzyme. The utility of this method is exemplified by the positive analysis of serum samples from Japanese victims of the terrorist attack with sarin in the Tokyo subway in 1995.

Introduction

Organophosphates (OP[1] compounds) with anti-cholinesterase activity have been widely used as pesticides and incidentally also as chemical warfare agents ("nerve agents"). The high toxicity of the latter agents can be attributed to the excessive cholinergic stimulation caused by inhibition of acetylcholinesterase (AChE).[1] With the recent proliferation of terrorism, rapid, sensitive, and reliable methods are required for retrospective detection of exposure to and handling of these agents.

Basically, three approaches have been explored to detect exposure to an OP compound. The oldest method comprises measurement of AChE activity in blood. The original colorimetric Ellman procedure (*1*) is generally used for occupational health screening and therapeutic monitoring of pesticide-poisoned patients. It suffers from serious drawbacks, since (i) it does not identify the OP compound, (ii) the specificity of the method is low, i.e., the interference with various unrelated chemicals is high, (iii) it does not provide reliable evidence for exposures at inhibition levels less than 20%, which is due to both substantial intra- and interindividual variations while control activity levels are often not available, and (iv) it is less suitable for retrospective detection of exposure due to de novo synthesis of enzyme.

A second method is based on measurement of the hydrolysis products of nerve agents, e.g., *O*-alkyl methylphosphonic acids. Methods for analysis of these compounds are based on GC-MS (*2*−*4*) or on LC-MS (*5*−*8*). The advantage of determination of hydrolysis products, compared to the first method, is the partial identification of the nerve agent. A serious drawback, however, is the rather rapid elimination rate of the hydrolysis product from the organism (within several days), which limits its use for retrospective detection of exposure.

The third method relies on analysis of modified human butyrylcholinesterase (HuBuChE),[1] an enzyme closely related to human AChE. Nerve agents react rapidly with the serine-198 residue in the active site, under formation of a phosphate or phosphonate ester. Rather long half-life times for this enzyme have been reported, ranging from 5−16 days (*9*−*11*), and its concentration in plasma is approximately 80 nM (*12*), making it a persistent and abundant source for biomonitoring of exposure to OP anticholinesterases. Our group (*13*) developed a procedure for analysis of phosphylated HuBuChE in plasma or serum samples, which is based on reactivation of the phosphylated enzyme with fluoride ions: this converts the OP moiety quantitatively into the corresponding phosphofluoridate, which is subsequently isolated and quantified by GC-NPD or GC-MS. A more or less related approach was reported by Nagao et al. (*14*) and Matsuda et al. (*15*), which is based on isolation and trypsinization of inhibited AChE, subsequent treatment with alkaline phosphatase, and finally, isolation, derivatization, and GC-MS analysis of the released phosphyl moiety. Unfortunately, this method is rather laborious, and it has the drawback that AChE is much less abundant than HuBuChE in human blood. The advantages of these methods are that both the origin and the extent of the OP poisoning can be determined. Furthermore, in contrast with the intact agent or its metabolites, the in vivo lifetime of phosphyl moieties bound to HuBuChE is limited only by (i) in vitro sequestration of the inhibited enzyme, (ii) spontaneous reactivation, and (iii) aging of the inhibited enzyme (i.e., loss of the alkyl moiety from the phosphyl group). Aging of the inhibited enzyme blocks the reactivation reaction with fluoride ions, since attack of a fluoride ion at the negatively charged phosphonate moiety is highly unfavorable. Thus, after treatment with fluoride ions of human plasma which had been exposed to soman (known to age very rapidly) in vitro, the regeneration of soman could not be detected (Van der

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[1] Abbreviations: AChE, acetylcholinesterase; Boc, *tert*-butyloxycarbonyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Fmoc, 9-fluorenylmethoxycarbonyl; HuBuChE, human butyrylcholinesterase; IMPA, isopropyl methylphosphonic acid; MPA, methylphosphonic acid; OP, organophosphate; PB, pyridostigmine bromide; TFA, trifluoroacetic acid.

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Schans, unpublished results). Nevertheless, the aged inhibited enzyme which contains a methylphosphonic acid moiety is still a potential target for biomonitoring of nerve agent exposure.

In this paper, we present a novel and general procedure for detection of exposure to nerve agents, which surpasses the limitations of the fluoride reactivation method. This method is based on the mass spectrometric analysis of phosphylated nonapeptides resulting after digestion of (aged and nonaged) inhibited HuBuChE with pepsin. We demonstrate that this method can be applied for unambiguous detection of in vivo exposures to nerve agents, as evidenced by the analysis of serum samples obtained from Japanese victims of the terroristic attack with sarin in the Tokyo subway in 1995. Furthermore, it will be shown that this method has the potential to be broadly applicable for detection of exposures to a wide range of HuBuChE binding pesticides.

Materials and Methods

Chemicals. Purified human butyrylcholinesterase (HuBuChE, EC 3.1.1.8) was obtained from Dr. B. P. Doctor of the Walter Reed Hospital, Washington DC. Procainamide-Sepharose 4B gel (capacity 29 μmol/mL was a kind gift of Dr. Y. Ashani, Israel Institute for Biological Research (Ness Ziona, Israel). Trifluoroacetic acid (TFA),[1] Tris(hydroxymethyl)-aminomethane hydrochloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Acros (Geel, Belgium). Pyridostigmine bromide (PB),[1] butyrylthiocholine iodide, TPCK treated trypsin (EC 3.4.21.4), thermolysin (protease type X; EC 3.4.24.27) and Pronase (protease type XIV from *Streptomyces griseus*, EC 3.4.24.31) were purchased from Sigma (Zwijndrecht, The Netherlands). Dimethylparaoxon and diethylparaoxon were purchased from Dr. S. Ehrenstorfer (Augsburg, Germany). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB),[1] sodium dodecyl sulfate (SDS) was purchased from Aldrich (Zwijndrecht, The Netherlands). Dithiothreitol (DTT)[1] and sodium iodoacetate were purchased from Fluka (Zwijndrecht, The Netherlands). Pepsin (EC 3.4.23.1) was purchased from Roche (Almere, The Netherlands). N-glycosidase F was purchased from Boehringer Mannheim (Almere, The Netherlands). Phosphate buffered saline (PBS) was purchased from BioWhittaker (Verviers, Belgium). All buffers were prepared with Millipore grade water.

Instrumentation. HPLC was performed using a Gilson (Villers-le-Bel, France) HPLC system consisting of a 305 master pump, a 306 slave pump, an 805 manometric module and an 811C dynamic mixer. Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm × 150 mm; 5 μm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden). The mobile phase consisted of a linear gradient (0′–20′ min) of 0.1% (v/v) trifluoroacetic acid (TFA)[1] in water to 48% (v/v) acetonitrile and 52% (v/v) water with 0.1% (v/v) TFA. The LC flow was 1 mL/min. The eluate was monitored at 214 nm with a Spectroflow 757 UV detector (Applied Biosystems, Ramsey, NJ) and with a radiometric detector (Radiomatic, model Flo-one Beta series A 500, Meriden, CT) with Ultima-Flo (Packard, Meriden, CT) as scintillation cocktail. Liquid scintillation countings were performed with a A2500 TR scintillation counter (Packard) with Hionic Fluor (Packard) as scintillation cocktail. UV-spectroscopy was performed on a Lambda 400 spectrophotometer (Perkin-Elmer, Uberlingen, Germany).

Peptides were synthesized on an Abimed (Langenfeld, Germany) AMS 422 peptide synthesizer, applying standard 9-fluorenylmethoxycarbonyl (Fmoc)[1] chemistry, using commercially available building blocks (Novachem, Läufelfingen, Switzerland). Slide-a-Lyzer cassettes (0.1–0.5 mL) were obtained from Pierce (Rockford, IL). Centrex UF-2 (3 or 10 kDa molecular weight cutoff) centrifugal ultrafilters were procured fro Schleicher & Schuell (Keene, NH). Ultrafree (100 kDa molecul weight cutoff; 15 mL) centrifugal ultrafilters were obtained fro Millipore (Bedford, MA).

Electrospray LC/Tandem MS Analysis. All mass spectr metric experiments were conducted on a Q-TOF hybrid instr ment equipped with a standard Z-spray electrospray interfac (Micromass, Altrincham, U.K.) and an Alliance, type 2690 liqui chromatograph (Waters, Milford, MA). The chromatographi hardware consisted of a precolumn splitter (type Acurate; L Packings, Amsterdam, The Netherlands), a sixport valve (Valco Schenkon, Switzerland) with a 10 or 50 μL injection loo mounted and a PepMap C_{18} (LC Packings) or Vydac C18 colum (both 15 cm × 300 μm i.d., 3 μm particles). A gradient of eluent A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation, following 100% (v/v) A (at time 0, 0.1 mL/min flow) to 100% (v/v) A (at 5 min, 0.6 mL/min flow) to 100% (v/v) B (at 45 min, 0.6 mL/min flow) or to 20% (v/v) A and 80% (v/v) B (at 60 min, 0.6 mL/min). The flow delivered by the liquid chromatograph was split precolumn to allow a flow of approximately 6 μL/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 V, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Synthesis of [methyl-^{14}C]Soman. For the preparation of [methyl-^{14}C]soman the method of Ott (*16*) was followed, with a few modifications. Thus, reaction of [^{14}C]CH_3I (150 mCi; 53 mCi/mmol) with Tris-(trimethylsilyl)phosphite (instead of triphenyl phosphite, as used by Ott) gave, after hydrolysis, [^{14}C]methylphosphonic acid which was converted into [^{14}C]methylphosphonic dichloride with thionyl chloride. Subsequent conversion into the difluoride was accomplished with KF. Finally, the pinacolyl group was introduced by reaction with pinacolyl alcohol under the agency of triethylamine. Radiochemical purity, as determined with HPTLC analysis on silica gel [eluent: 5% (v/v) methanol in chloroform] > 95%. Specific activity: 53 mCi/mmol. Overall radiochemical yield: 25%.

Activity Measurements of HuBuChE. HuBuChE activities were determined with the Ellman method (*1*). Briefly, the absorption was read at 412 nm after incubation of a HuBuChE containing sample with a mixture of butyrylthiocholine (0.8 mM) and DTNB (0.8 mM) for 5 min at 25 °C.

Incubation of HuBuChE with [methyl-^{14}C]Soman. A solution of purified HuBuChE (1 mg) in PBS (100 μl) was incubated with [methyl-^{14}C]soman (approximately 5 equiv) for 3.5 h at 37 °C. Next, the solution was dialyzed against PBS (2 × 1 L) overnight at 4 °C, to remove the excess of [methyl-^{14}C]-soman. In case reduction/carboxymethylation of HuBuChE was performed (vide infra), the dialysis buffer consisted of 5 M urea, 0.5 M Tris.HCl (pH 8.5; temperature of calibration 20 °C) instead of PBS.

Reduction and Carboxymethylation of HuBuChE. To dialyzed [methyl-^{14}C]soman inhibited HuBuChE (0.7 mg) in 5 M urea/0.5 M Tris.HCl (pH 8.5; temperature of calibration 20 °C; 350 μl), DTT (2.2 mg) was added and the mixture was incubated for 60 min at 50 °C. Next, sodium iodoacetate (5 mg) was added and the mixture was incubated for 60 min at 50 °C. The mixture was dialyzed against aqueous NH_4HCO_3 (50 mM; 2 × 1 L) overnight at 4 °C.

Deglycosylation of HuBuChE. Reduced and carboxymethylated [methyl-^{14}C]soman inhibited HuBuChE (4.5 nmol; 0.36 mg) in NH_4HCO_3 buffer (50 mM; 300 μL) containing 0.01% (w/w) SDS was incubated overnight at 37 °C with N-glycosidase F (8 μL of a solution of 100 units N-glycosidase F in 100 μL 50 mM NH_4HCO_3).

Digestion of HuBuChE with Trypsin. A solution of pretreated [methyl-^{14}C]soman-inhibited HuBuChE (reduced, carboxymethylated and deglycosylated; 0.25 mg) in aqueous NH_4HCO_3 (50 mM; 100 μL) was incubated with TPCK treated trypsin (2%, w/w) at 37 °C. At various time points samples were

taken for HPLC analysis with radiometric detection, using a Source 15 RPC column, and for electrospray LC/tandem MS analysis.

Digestion of HuBuChE with Thermolysin. A solution of pretreated [methyl-^{14}C]soman-inhibited HuBuChE (reduced, carboxymethylated and deglycosylated; 0.25 mg) in a buffer containing 60 mM KCl, 2 mM NaCl, 6 mM $CaCl_2$, 60 mM Tris.HCl (pH 7.5; temperature of calibration 20 °C; 200 μL), was incubated with various amounts of thermolysin (3 to 10%, w/w) at 37 °C. At various time points samples were taken for HPLC analysis with radiometric detection, using a Source 15 RPC column, and for electrospray LC/tandem MS analysis.

Digestion of HuBuChE with Pronase. A solution of pretreated [methyl-^{14}C]soman-inhibited HuBuChE (reduced, carboxymethylated and deglycosylated; 0.25 mg) in aqueous NH_4HCO_3 (50 mM; 200 μL) was incubated with Pronase (5%, w/w) at 37 °C. At various time points samples were taken for HPLC analysis with radiometric detection and for electrospray LC/tandem MS analysis.

Digestion of HuBuChE with Pepsin. [methyl-^{14}C]Soman-inhibited HuBuChE (72 μg) was incubated with pepsin (1.5% − 150% w/w) in 5% (v/v) aqueous formic acid (530 μL) for 2 h at 37 °C. At various time points samples were taken for HPLC analysis with radiometric detection on a Source 15 RPC column. At various time points samples were filtrated through 3 kDa MW cutoff filters. The filtrate was analyzed by means of HPLC analysis with radiometric detection, using a Source 15 RPC column, and by means of electrospray LC/tandem MS analysis.

Isolation of HuBuChE from Serum and Pepsin Digestion. An LPLC system (Pharmacia, Uppsala, Sweden), consisting of two peristaltic pumps (P-1), a gradient controller (GP-250), a fraction collection module (Frac-100) and UV detector (UV-1; 254 nm), was used for the procainamide affinity chromatography to isolate HuBuChE from plasma (*17*). Serum samples (0.1−1 mL) were applied on the column [1.7 cm (diameter) × 4 cm (length)], filled with 7−8 mL procainamide gel, which was eluted with buffer A (20 mM phosphate, 1 mM EDTA, pH 6.9) and B (20 mM phosphate, 1 mM EDTA, 600 mM sodium chloride, pH 6.9), applying the following gradient (all in v/v %): 0−10 min, 0% B to 16.7% B; 10−25 min, 16.7% B; 25−35 min, 16.7% B to 100% B; 35−60 min, 100% B; 60−65 min, 100% B to 100% A; 65−110, 100% A. Flow: 1 mL/min. Fractions were checked for the presence of HuBuChE activity by the Ellman procedure and radioactivity (in case of experiments with [methyl-^{14}C]soman) was determined by liquid scintillation. Column fractions containing radioactivity and/or residual HuBuChE activity were heated for 15 min at 95 °C. The formed precipitate was removed by centrifugation and the retentate was transferred to 100 kDa cutoff filters for concentration (until a volume of 0.2 mL) of HuBuChE by centrifugation at 3220*g*. The retentate was washed with aqueous NH_4HCO_3 (50 mM; 2 × 10 mL) and subsequently transferred in aqueous NH_4HCO_3 (50 mM; 600 μL) to a 3 kDa filter and concentrated again. To this concentrate, a solution of pepsin (0.02% w/v) in 5% (v/v) formic acid (300 μL) was added and the mixture, which was still on the filter, was incubated for 2 h at 37 °C. After filtration (3220*g*) and washing of the retentate with 5% (v/v) aqueous formic acid (2 × 200 μL) the sample was concentrated under reduced pressure, redissolved in 5% (v/v) formic acid (50−100 μL) and analyzed with electrospray LC/tandem MS.

Synthesis of Phosphonylated Peptides. Boc^1-FGES*AGAAS, with S* an unprotected serine residue, was synthesized on a Tentagel resin, by applying standard Fmoc chemistry. In case of FGE(S-MPA)AGAAS, with S-MPA^1 a serine-methylphosphonic acid residue, on-resin phosphorylation was carried out according to Wijkmans et al. (*18*) using *O*-p-methoxybenzyl bis(di-isopropyl)phosphoamidite as the phosphitylating reagent. The synthesis of FGE(*S*-IMPA)AGAAS, with *S*-IMPA^1 a serine-(*O*-isopropyl)methylphosphonic acid residue, was carried out analogously, using *O*-isopropyl bis(di-isopropyl)-phosphoamidite as the phosphytilating agent. After cleavage from the resin and removal of protecting groups the desired phosphonylated peptides were obtained in good yield and purity. HPLC and mass spectrometrical data were in accordance with the phosphonylated peptides found in pepsin digests.

Results

We explored several proteolytic enzymes in order to develop a rapid and sensitive assay for mass spectrometric analysis of phosphylated HuBuChE.

Digestion with Trypsin. Nachon et al. (*19*) reported in their studies on the quaternary ammonium binding sites in HuBuChE that pretreatment by S-carboxymethylation and deglycosylation was required for proper trypsination of HuBuChE. Thus, when pretreated [methyl-^{14}C]soman-inhibited HuBuChE was incubated with trypsin for 2 h, one major radioactive peak was observed upon analysis by means of HPLC with radiometric detection.[2] This peak was characterized as SVTLFGE-(*S*-[^{14}C]MPA)AGAASVSLHLLSPGSHSLFTR with *S*-[^{14}C]-MPA representing the serine-198 residue conjugated to [^{14}C]MPA. The transformed electrospray tandem MS spectrum is shown in Figure 1. After prolonged incubation this peak was slowly converted into a peak with a slightly longer retention time which was identified as SVTLFGE(*S*-[^{14}C]MPA)AGAASVSLHLLSPGSHSLF, probably resulting from chymotrypsin activity. The tryptic fragment, containing the serine-198 residue conjugated to *O*-pinacolyl methylphosphonic acid (MH_3^{3+} 1031.8), resulting from nonaged phosphonylated HuBuChE, could also be detected in a ratio 3/1 (aged/nonaged). As expected, the same results were obtained when nonlabeled soman was used.

Digestion with Protease Type X (Thermolysin). After digestion with thermolysin, which normally cleaves at the amino side of leucine and phenylalanine, a much smaller phosphonylated peptide should result which might have more beneficial mass spectrometric properties. As expected, no radioactive peptide material was detected without pretreatment of HuBuChE. On the other hand, when pretreated, inhibited HuBuChE was incubated with thermolysin several radioactive peptides were formed according to HPLC with radiometric detection. After 20 h, the major peak in the chromatogram represented 60% of total bound radioactivity (results not shown). This peak was collected and identified as FGE-(*S*-[^{14}C]MPA)AG on the basis of the product ion spectrum of MH^+ with *m/z* 647.3.

Digestion with Pronase. Pronase is a protease that randomly cuts proteins to amino acids and small peptides. Again, we did not observe any substantial digestion without pretreatment of HuBuChE. When pretreated inhibited HuBuChE was incubated with Pronase for 4 h, HPLC with radiometric detection showed the presence of 3 radioactive peaks. The major peak (approximately 60% of totally bound radioactivity) was identified as GE-(*S*-[^{14}C]MPA)AGAA (MH^+ 642.2) with electrospray LC/tandem MS. The identity of the other peaks has not been elucidated. Incubation for longer times did not significantly alter the chromatogram.

Digestion with Pepsin. Pepsin requires acidic conditions to work properly (optimum pH 1.8−2.2) and cleaves preferentially bonds at the carboxylic side of Phe, Met, Leu or Trp. Thus, [methyl-^{14}C]soman inhibited HuBuChE

[2] When it was attempted to trypsinize [^{14}C]soman inhibited HuBuChE directly, it could be deduced from the HPLC chromatograms that hardly any digestion had occurred.

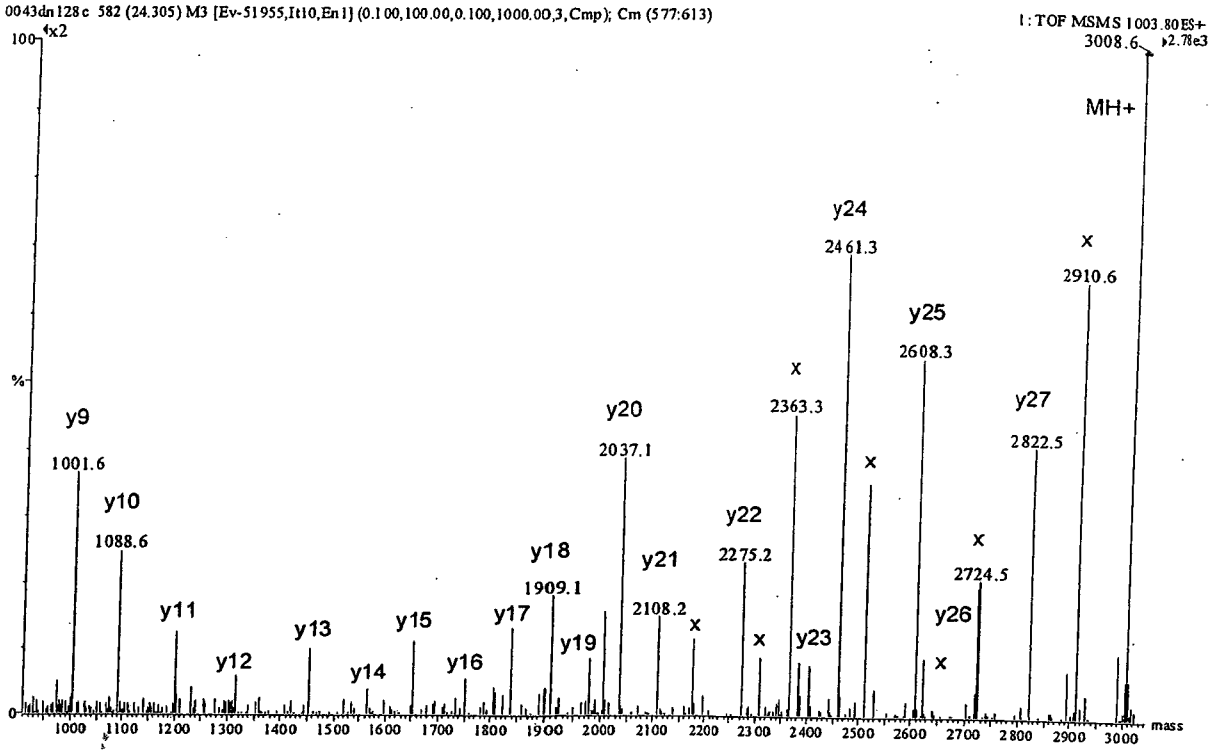

Figure 1. Part of the transformed spectrum (MaxEnt3 software) of the modified tryptic fragment of SVTLFGE(S-[^{14}C]MPA)-AGAASVSLHLLSPGSHSLFTR, with *S*-[^{14}C]MPA representing the serine-198 residue conjugated to methylphosphonic acid, in a tryptic digest from human butyrylcholinesterase exposed to 5 equiv of [methyl-^{14}C]soman. Fragments with "x" represent the loss of [^{14}C]MPA from MH^+ 3008.6 and from the fragments y_{27} to y_{22}, respectively.

(without pretreatment) was digested with 1.5% (w/w) pepsin in 5% (v/v) formic acid. According to HPLC with radiometric detection, one radioactive peak resulted, which was identified as the dodecapeptide FGE(*S*-[^{14}C]-MPA)AGAASVSL by means of electrospray LC/tandem MS (MH^+ 1174.5), which is in accordance with the results obtained by Lockridge (*20*) for native HuBuChE. After incubation for 2 h, digestion had progressed for 65%. In an attempt to accelerate the procedure, digestion was performed with 15% (w/w) and 150% (w/w) pepsin. The HPLC analyses of the digests (Figure 2) indicate that a different peptide forms when larger amounts of pepsin are used; upon closer inspection of the HPLC chromatogram this peak contains a shoulder (estimated 20−30% of total area). The main peptide was identified with electrospray LC/tandem MS as the nonapeptide FGE(*S*-[^{14}C]MPA)AGAAS (MH^+ 876.3; Figure 3) while the shoulder was identified as the octapeptide FGE(*S*-[^{14}C]-MPA)AGAA (MH^+ 789.3). In the same digest a conjugate of the nonapeptide with O-pinacolyl methylphosphonic acid was detected (10% of main peak; MH^+ 960.6). Synthetic FGE(*S*-[^{14}C]MPA)AGAAS coeluted with the major radioactive peak and gave identical, but isotope shifted mass spectrometric data as the major adduct peak detected in the digest. When incubation (48 h) was prolonged, the HPLC chromatogram did not change significantly. On the other hand, when incubation with 1.5% (w/w) pepsin was prolonged (48 h), the dodecapeptide was converted into the nonapeptide.

Mass Spectrometric Analysis of Methylphosphonylated HuBuChE Isolated from Plasma. On the basis of the abovementioned results, we selected pepsin digestion as the method of choice for analyzing methylphosphonylated HuBuChE in human plasma. Plasma was incubated with [methyl-^{14}C]soman and HuBuChE

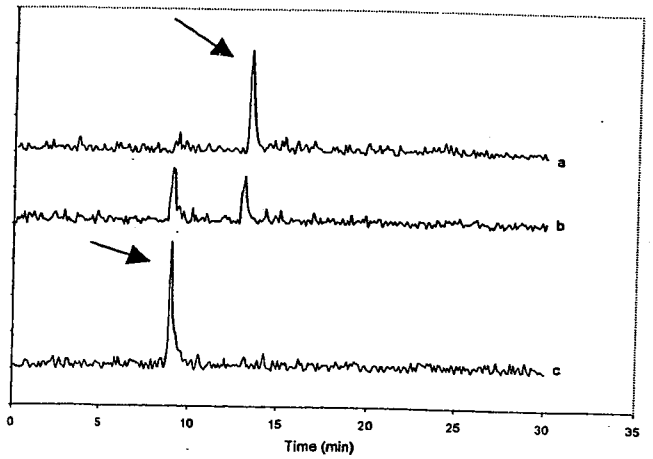

Figure 2. HPLC analysis (Source 15RPC column) of a pepsin digest of HuBuChE exposed to 5 equiv of [methyl-^{14}C]soman. Digestion was performed for 2 h at 37 °C, using various amounts of pepsin. (a) Digestion with 1.5% w/w; (b) digestion with 15% w/w; (c) digestion with 150% w/w. The eluent (flow rate of 1 mL/min) was 0.1% TFA in H_2O with a linear gradient to 0.1% TFA in CH_3CN/H_2O (48/52, v/v) over the course of 20 min. The arrow in the upper trace indicates the peak for FGE(*S*-[^{14}C]-MPA)AGAASVSL and the arrow in the lower trace denotes the peak for FGE(*S*-[^{14}C]MPA)AGAAS.

was isolated using procainamide affinity chromatography (*17*). Fractions were checked for presence of HuBuChE by the Ellman procedure and radioactivity was determined by liquid scintillation counting. Maximum HuBuChE residual activity coeluted with maximum radioactivity. For further cleanup, the pooled and partly concentrated fractions were heated for 15 min at 95 °C, after which precipitated proteins were removed by centrifugation. It was established that no radioactivity was present in the residue. After pepsin digestion and work-

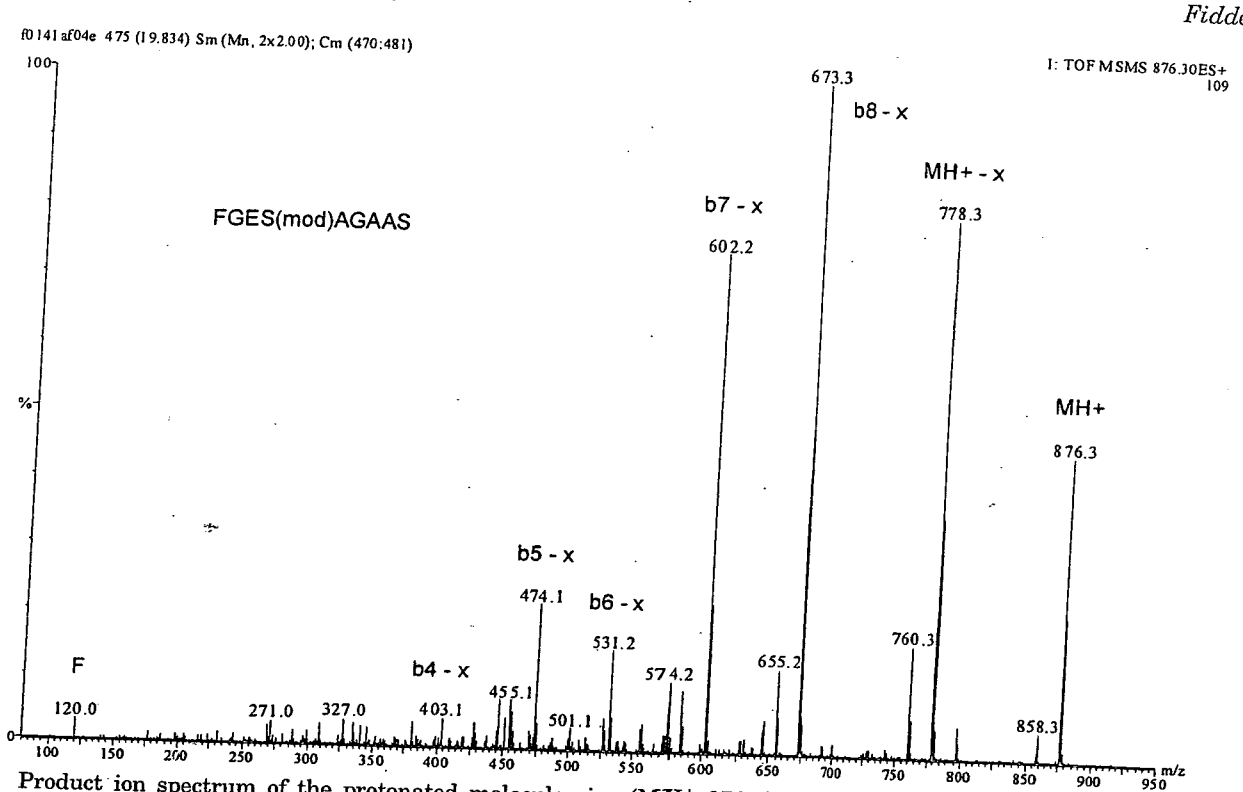

Figure 3. Product ion spectrum of the protonated molecular ion (MH^+ 876.3) of FGE(*S*-[^{14}C]MPA)AGAAS, with *S*-[^{14}C]MPA representing the serine-198 residue conjugated to methylphosphonic acid, in a pepsin digest from human butyrylcholinesterase exposed to 5 equiv of [methyl-^{14}C]soman; "x" = [^{14}C]MPA.

up, electrospray LC/tandem MS analysis demonstrated the presence of FGE(*S*-[^{14}C]MPA)AGAAS with MH^+ 876.3. When human plasma was incubated with various amounts of soman, a more or less linear relationship between inhibition level and amount of FGE(*S*-[^{14}C]-MPA)AGAAS was observed. However, care has to be taken, since no internal standard was used. The recovery of the procedure was determined to be 50−60% (based on duplicate runs), as established by workup of [methyl-^{14}C]soman inhibited HuBuChE.

We were curious to find out whether the same assay could be used for detection of exposure to other nerve agents. Pepsin digestion of purified HuBuChE exposed to *O*-isopropylmethylphosphonofluoridate (sarin) led to the formation of the corresponding *O*-isopropyl methylphosphonic acid (IMPA)[1] nonapeptide and the corresponding octapeptide in a similar ratio as that obtained earlier for soman. The IMPA-nonapeptide coeluted with the synthetic standard and exhibited identical mass spectrometric data. The procedure could also successfully be applied to HuBuChE isolated from plasma which had been exposed to sarin.

Mass Spectrometric Analysis of (*O*-isopropyl)-Methylphosphonylated HuBuChE in Japanese Serum Samples. At this stage, it was investigated whether the assay could be used for biomonitoring of "real" samples, i.e, samples resulting from accidental and/or intentional in vivo exposures. Recently, we published results on the analysis of serum samples from victims of terrorist attacks in the Tokyo subway and in Matsumoto (*8, 13*). Serum samples from victims of the Tokyo attack had been taken within 1.5 h after the incident, were kindly donated to TNO Prins Maurits Laboratory and stored since then at −70 °C. Two of these samples (0.3 and 0.5 mL) were worked up (single experiments, due to the small sample size) and analyzed (single analysis in case of the 0.3 mL sample; duplicate analyses in case of the 0.5 mL sample) as described above. In Figure 4 the results of one of these analyses are shown. Electrospray LC/tandem MS analyses were carried out by selecting MH^+ (*m/z* 916.3) in the first mass spectrometer, and monitoring one of the main fragment ions (*m/z* 778.4) in the second MS. As can be derived from Figure 4, the blank sample was clean at the *m/z* 778 trace (trace a), and the synthetic standard (trace c) coeluted with the observed peak in the Japanese serum samples (trace b). For the electrospray tandem MS spectrum of the adduct detected in this sample, see Figure 5. The detection limit for the IMPA-nonapeptide was determined to be 150 pg for a stock solution in water. At these levels, no matrix effects were observed for analyses in plasma samples. In the Japanese samples, the IMPA-nonapeptide was analyzed at levels of 5 and 10 pmol/ml of processed serum, based on comparison with a blank serum sample which had been spiked, after workup, with a well-defined amount of the synthetic standard. Consequently, based on 50% recovery for workup and digestion, this implies a level of 10 or 20 pmol inhibited HuBuChE/ml serum.

Mass Spectrometric Analysis of HuBuChE Modified by Other Agents. It was investigated whether the assay could also be used for detection of exposure to OP pesticides. Thus, purified HuBuChE was inhibited with dimethylparaoxon and diethylparaoxon (5 equiv in both cases) and subsequently, together with soman and sarin-inhibited HuBuChE, digested with pepsin. The digests were mixed and analyzed with electrospray LC/tandem MS (see Figure 6); the corresponding MS/MS spectra are shown in Figure 7. The assay also proved to be suitable for analysis of HuBuChE, modified by non-OP anticholinesterases. Thus, after incubation of HuBuChE with pyridostigmine bromide (PB),[1] the carbamoylated nonapeptide (MH^+ 867.3; see Figure 8) could be detected in the pepsin digest.

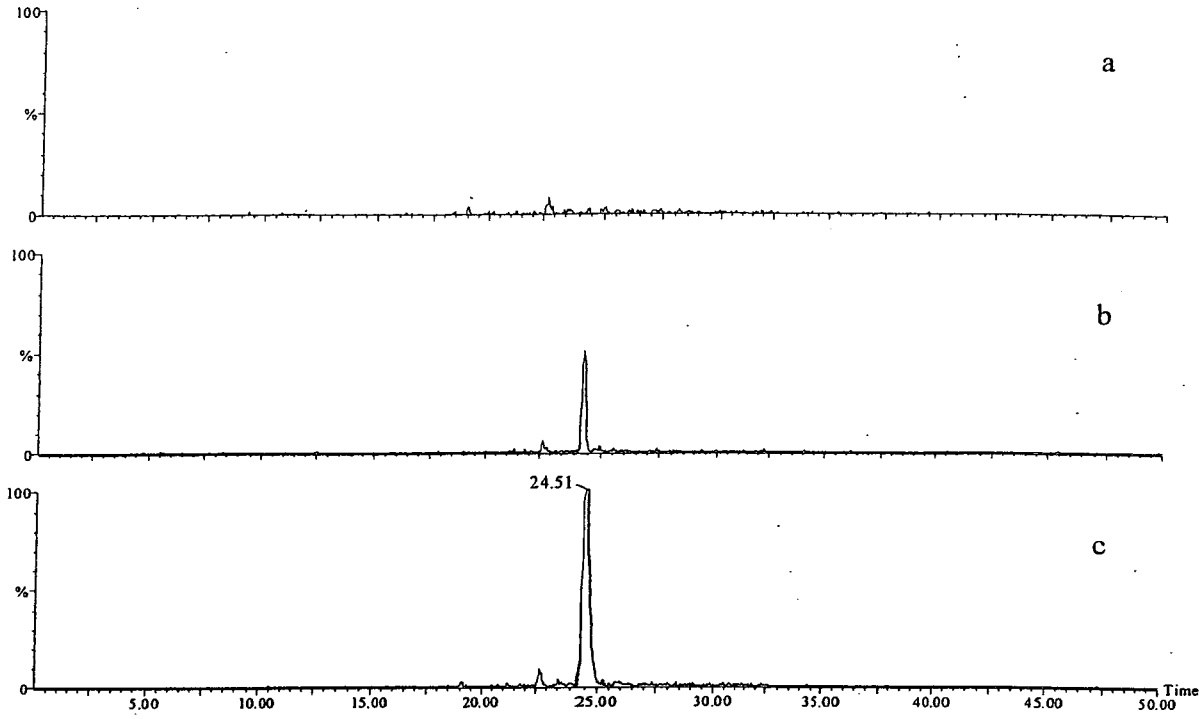

Figure 4. Ion chromatograms of fragment ion *m/z* 778.4 originating from FGE(*S*-IMPA)AGAAS, with *S*-IMPA representing the serine-198 residue conjugated to O-isopropyl methylphosphonic acid, in a pepsin digest of human butyrylcholinesterase (HuBuChE). HuBuChE was isolated from serum of nonexposed human blood (a) and from a Japanese victim of the terroristic attack with sarin in the Tokyo metro (b). Trace c represents the pepsin digest shown in trace a, after spiking with synthetic FGE(*S*-IMPA)AGAAS.

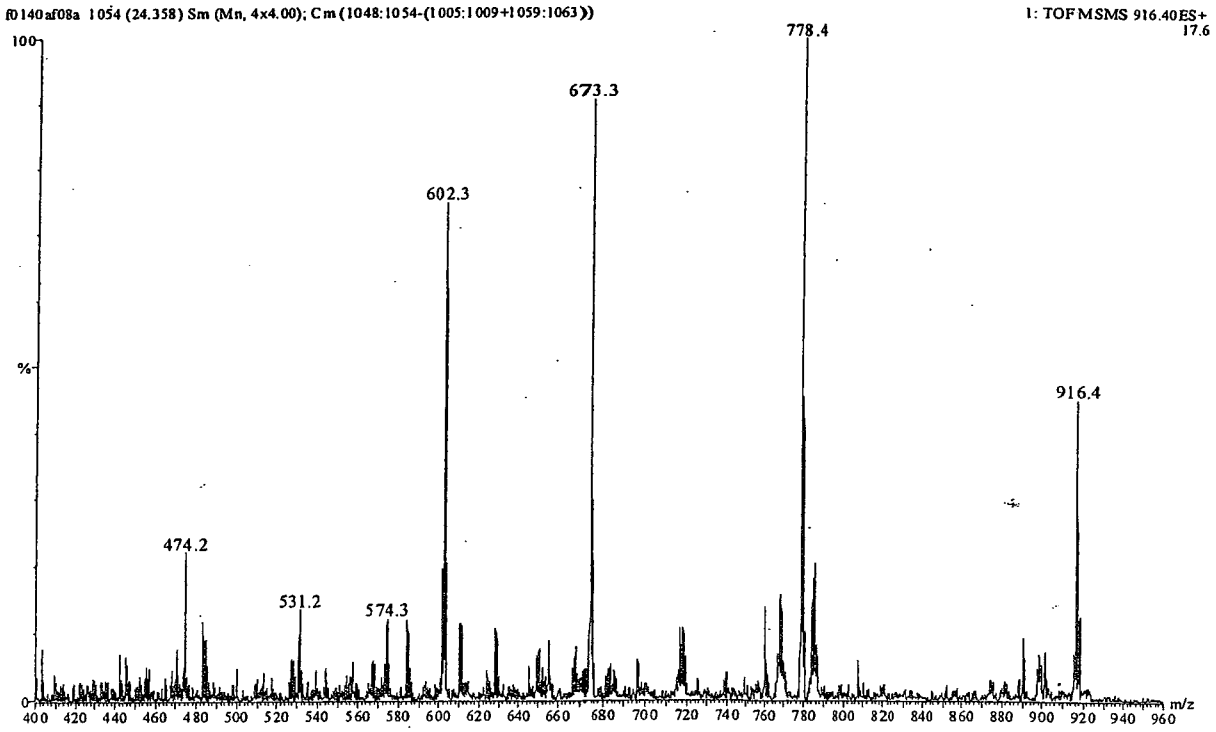

Figure 5. Product ion spectrum of the protonated molecular ion (MH^+ 916.4) of FGE(*S*-IMPA)AGAAS, with *S*-IMPA representing the serine-198 residue conjugated to O-isopropyl methylphosphonic acid, in a pepsin digest from human butyrylcholinesterase isolated from serum of a Japanese victim of the terroristic attack with sarin in the Tokyo metro.

Discussion

As part of a program aimed at the development of a general method for retrospective detection of exposure to nerve agents, and in particular a method suitable for detection of exposure to rapidly aging agents, we initiated a study toward the mass spectrometric analysis of HuBuChE, phosphylated at the serine-198 residue. The advantage of such an approach is that the adduct of the nerve agent with one of its main targets is analyzed, thus providing ultimate evidence for an alleged exposure. Recently, an analogous approach was described by Doorn et al. (*21*, *22*) and also by Elhanany et al. (*23*) in order to elucidate the mechanism of AChE inhibition by various OP compounds.

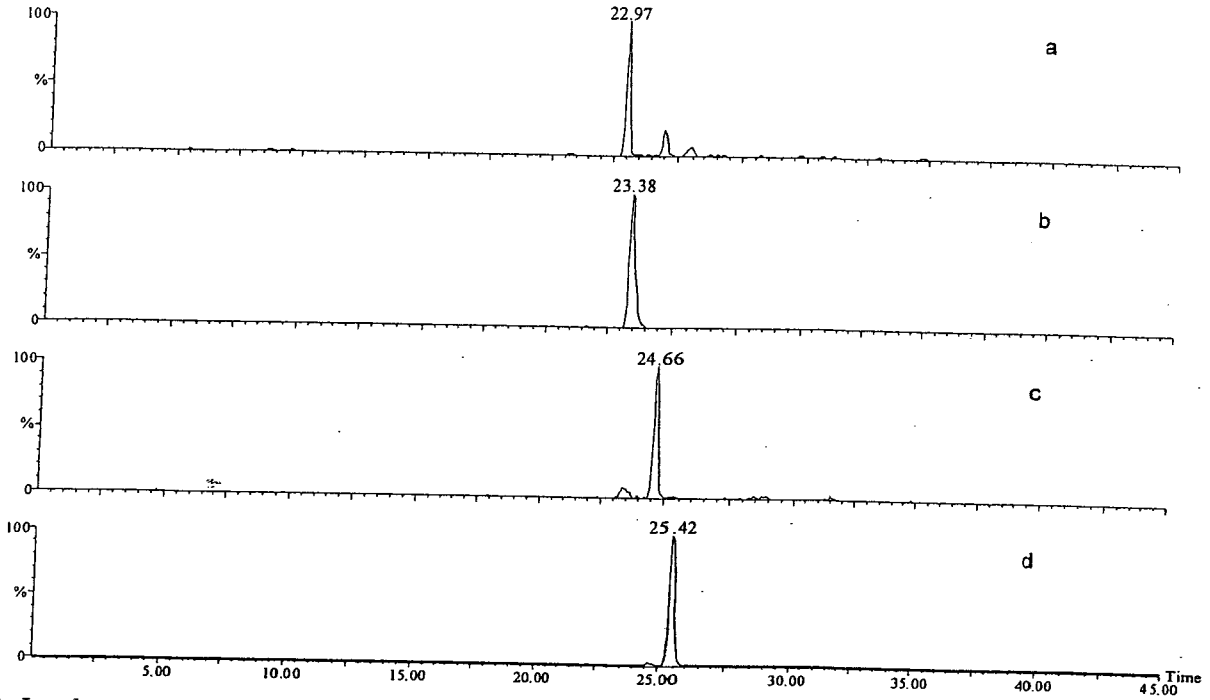

Figure 6. Ion chromatograms of fragment ion *m/z* 778.4 originating from FGE(S*)AGAAS, with S* representing the phosphylated serine-198 residue in a pepsin digest of a mixture of human butyrylcholinesterase (HuBuChE) inhibited with dimethylparaoxon (a), soman (b), sarin (c), and diethylparaoxon (d).

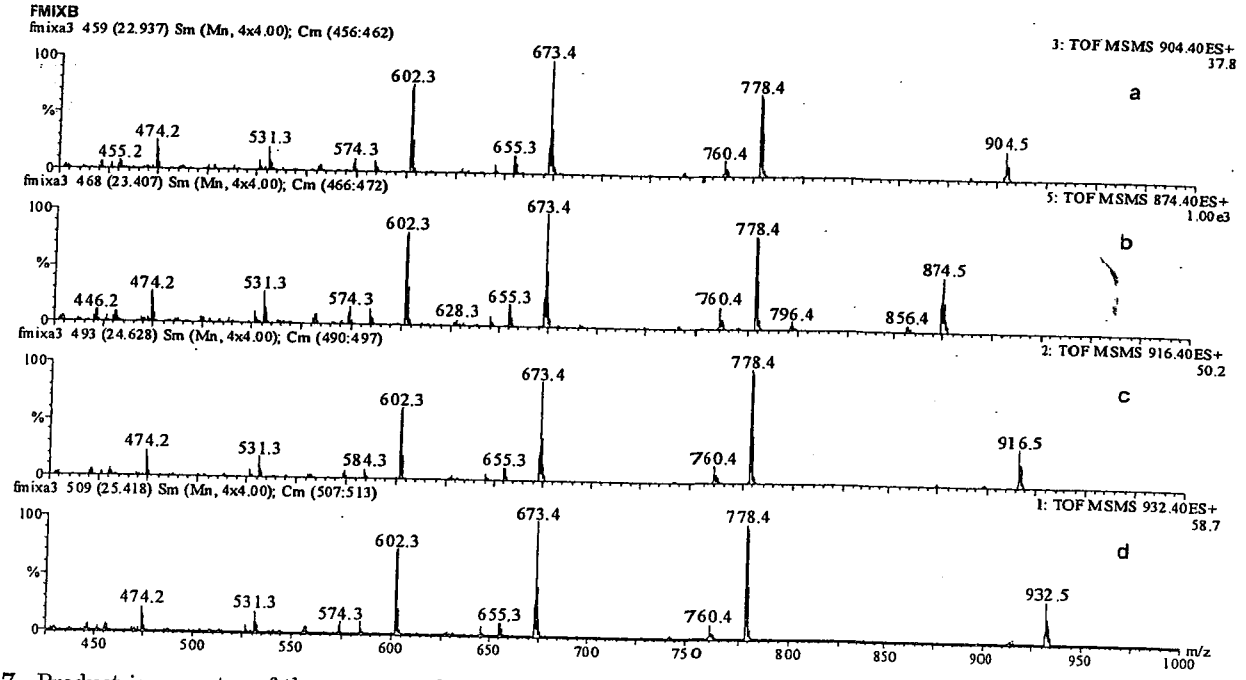

Figure 7. Product ion spectra of the protonated molecular ions of various FGE(S*)AGAAS, with S* representing the serine-198 residue conjugated to methylphosphonic acid, in a pepsin digest from human butyrylcholinesterase inhibited with 5 equiv of dimethylparaoxon (a), soman (b), sarin (c), and diethylparaoxon (d).

Several proteolytic enzymes were explored. Digestion with trypsin was rather laborious because pretreatment (carboxymethylation and deglycosylation) was required prior to digestion. Moreover, it gave a rather large phosphonylated peptide (29 amino acids residues), which probably precludes sensitive detection, due to excessive fragmentation in the mass spectrometer. Interestingly, the tryptic fragment with the O-pinacolyl methylphosphonic acid group was detected at a level of approximately 5%. This fragment probably results from HuBuChE which had been inhibited by a soman stereoisomer which is less prone to aging. After thermolysin digestion, FGE(*S*-[^{14}C]MPA)AG was found as the major adducted peptide. After digestion with Pronase, electrospray LC/tandem MS analysis showed the presence of GE(*S*-[^{14}C]MPA)AGAA as the major fragment. Unfortunately, in both cases pretreatment of the enzyme was required for optimal digestion, resulting in rather laborious procedures. Moreover, digestion with thermolysin was rather slow.

In case of pepsin digestion of HuBuChE, pretreatment of the enzyme was not necessary. Digestion of purified

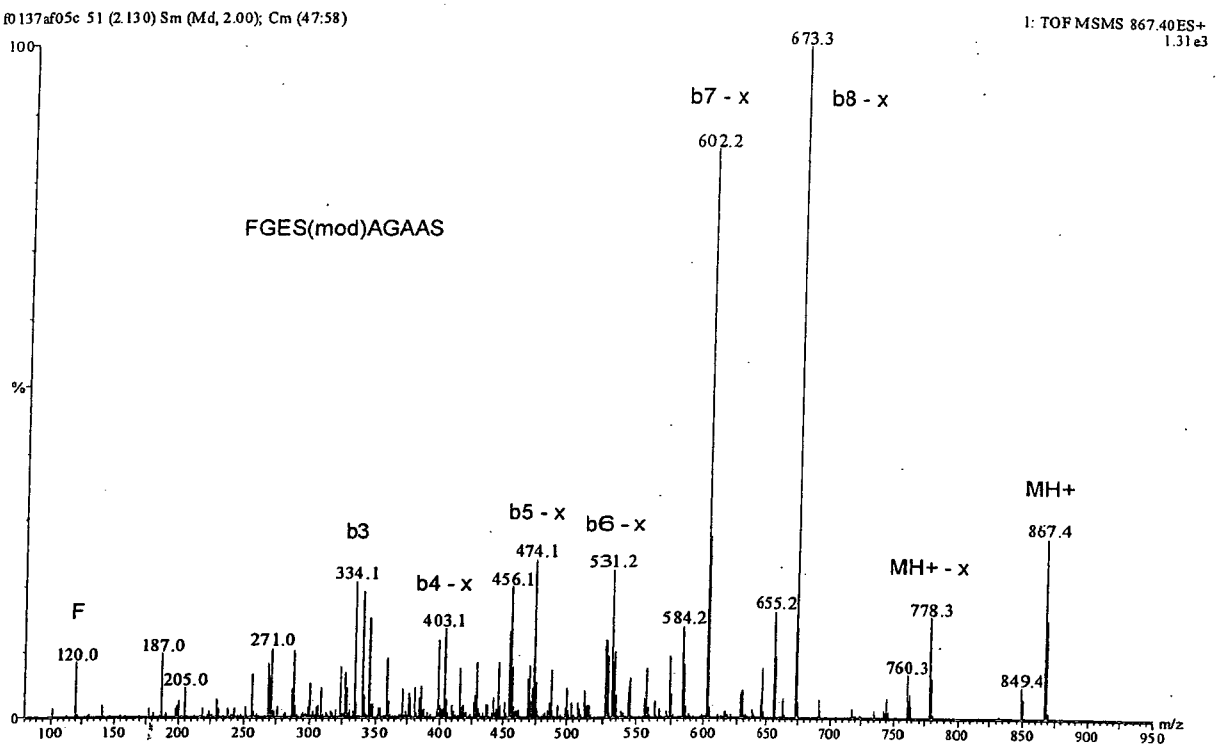

Figure 8. Product ion spectrum of the protonated molecular ion (MH^+ 867.4) of FGES*AGAAS, with S* representing the serine-198 residue conjugated to a dimethylcarbamoyl moiety, in a pepsin digest from human butyrylcholinesterase exposed to PB (1 equiv). "x" = $(CH_3)_2N$-COOH.

or crude HuBuChE samples isolated from plasma proceeded smoothly, under formation of a phosphylated nonapeptide FGE(*S*-[^{14}C]MPA)AGAAS, and to a lesser extent, the phosphylated octapeptide FGE(*S*-[^{14}C]-MPA)AGAA. Although digestion with 1.5% (w/w) resulted in the formation of the expected dodecapeptide FGE(*S*-[^{14}C]MPA)AGAASVSL (*20*), we found that the end product of the digestion was the abovementioned nonapeptide. Cleavage by pepsin after a serine residue has been observed earlier (*24*, *25*), though it is far less favorable than cleavage after Leu, Met, Phe, and Trp. The ratio nonapeptide/dodecapeptide was strongly dependent on the enzyme/substrate ratio: formation of the nonapeptide was highly accelerated by using a large excess of pepsin (150% w/w). Since the protein concentration in the processed samples resulting from procainamide chromatography is probably rather variable, we selected digestion with a large excess of pepsin for further experiments. In this respect, it was verified with HPLC with radiometric detection that the [^{14}C]MPA-nonapeptide was still the major fragment when HuBuChE isolated from plasma samples was digested with excess pepsin.

Phosphylated HuBuChE could readily be isolated from plasma by procainamide affinity chromatography indicating that inhibited HuBuChE has still affinity for immobilized procainamide, as was also reported by Masson et al. (*26*).

The procedure also worked for other agents having the serine-198 residue of HuBuChE as a target. Thus, the IMPA-nonapeptide could readily be detected in a pepsin digest of HuBuChE isolated from plasma which had been exposed to sarin. To demonstrate the utility of the method, two serum samples were analyzed, which had been taken from victims of the Tokyo subway terrorist attack with sarin in 1995. Electrospray LC/tandem MS analyses showed significant amounts of the IMPA-nonapeptide after isolation of HuBuChE and pepsin digestion. Control samples, which had been stored in close proximity to the Japanese samples in the same freezer compartment since arrival from Tokyo, were blank. The two samples have been analyzed earlier by using the fluoride reactivation procedure, in which case approximately 14 pmol of sarin/mL of serum was found (*13*). In this study, 10−20 pmol of inhibited HuBuChE/mL was determined, which is in the same order of magnitude. It has to be emphasized however that quantitation of these results is preliminary, because no internal standard has been used. Interestingly, the synthetic standard eluted as a relatively broad peak, while the IMPA-nonapeptide in the Japanese samples appeared as a sharp peak. This is probably caused by the fact that the synthetic standard is a mixture of two diastereomeric peptides due to the chirality of the phosphonate ester and, on the other hand, by the fact that mainly one sarin isomer (P(−) isomer) reacts with the enzyme, eventually resulting in a single isomer of the IMPA-nonapeptide.

The method appears to be suitable to detect HuBuChE modification by OP pesticides and non-OP anticholinesterases. Thus, in preliminary exposure experiments with dimethylparaoxon and diethylparaoxon, the corresponding nonapeptides were readily detected. The peaks of the various phosphylated nonapeptides were baseline separated, indicating that it is possible to determine exposure to mixtures of OP compounds, or subsequent exposures, in a single analysis run. A dimethylamine-carbamoylated nonapeptide was analyzed in case of exposure of HuBuChE to PB. This might allow mechanistic and toxicokinetic studies aimed at elucidation of the prophylactic effect exerted by PB for nerve gas intoxication. On the basis of this result, it is expected that the method

can also be used for detection of exposure to carbamate pesticides.

In conclusion, a general method has been developed which allows the unambiguous identification of serine-198 modified HuBuChE. This method can inter alia be used to detect exposure to nerve agents, such as the rapidly aging soman, and to OP pesticides. The utility of the method is exemplified by the analyses of serum samples from victims from the terroristic attack in Japan, indicating its importance for forensic analyses. Furthermore, the method should be applicable to detect and identify exposure to carbamate pesticides, in view of the results obtained for PB. The mass spectrometric analyses described in this paper were carried out on a Q-Tof mass spectrometer which enables the full scan tandem mass spectrometric identification of the modified peptides. However, more sensitivity should be attainable with a triple quadrupole instrument, which allows multiple reaction monitoring. This scanning mode will be the subject of future experiments.

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References

(1) Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961) A new rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.

(2) Shih, M. L., Smith, J. R., McMonagle, J. D., Dolzine, T. W., and Gresham, V. C. (1991) Detection of metabolites of toxic alkyl methylphosphonates in biological samples. *Biol. Mass Spectrom.* **20**, 717–723.

(3) Black, R. M., Clarke, R. J., Read, R. W., and Reid, M. T. J. (1994) Application of gas chromatography–mass spectrometry and gas chromatography-tandem mass spectrometry to the analysis of chemical warfare samples, found to contain residues of the nerve agent sarin, sulfur mustard and their degradation products. *J. Chromatogr. A* **662**, 301–321.

(4) Fredriksson, S.-Å., Hammarström, L.-G., Henriksson, L., and Lakso, H.-Å. (1995) Trace determination of alkyl methylphosphonic acids in environmental and biological samples using gas chromatography/negative-ion chemical ionization mass spectrometry and tandem mass spectrometry. *J. Mass Spectrom.* **30**, 1133–1143.

(5) Tørnes, J. A. (1996) Identification of some alkyl methylphosphonic acids by thermospray tandem mass spectrometry. *Rapid. Commun. Mass Spectrom.* **10**, 878–882.

(6) Black, R. M., and Read, R. W. (1997) Application of liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry, and tandem mass spectrometry, to the analysis and identification of degradation products of chemical warfare agents. *J. Chromatogr. A* **759**, 79–92.

(7) Black, R. M., and Read, R. W. (1998) Analysis of degradation products of organophosphorus chemical warfare agents and related compounds by liquid chromatography–mass spectrometry using electrospray and atmospheric pressure chemical ionisation. *J. Chromatogr. A* **794**, 233–244.

(8) Noort, D., Hulst, A. G., Platenburg, D. H. J. M., Polhuijs, M., and Benschop, H. P. (1998) Quantitative analysis of O-isopropyl methylphosphonic acid in serum samples of Japanese citizens allegedly exposed to sarin: estimation of internal dosage. *Arch. Toxicol.* **72**, 671–675.

(9) Hall, G. M., Wood, G. J., and Paterson, J. L. (1984) Half-life of plasma cholinesterase. *Br. J. Anaesth.* **56**, 903–904.

(10) Günther, R., Dönhardt, A., Altland, K., Jensen, M., and Goedde, J. W. (1971) Alkylphosphatintoxikation. Kasuistik einer schweren Vergiftung und Anwendung eines gerinigten menschlichen Cholinesterasepräparats. *Med. Klin.* **66**, 785–788.

(11) Munkner, T., Matzke, J., and Videbaek, A. (1961) Cholinesterase activity of human plasma after intramuscular diisopropyl fluorophosphonate (DFP). *Acta Pharmacol. Toxicol.* **18**, 170–174.

(12) Myers, D. K. (1952) Studies on cholinesterase-7: determination of the molar concentration of pseudo-cholinesterase in serum. *Biochem. J.* **51**, 303–311.

(13) Polhuijs, M., Langenberg, J. P. and Benschop, H. P. (1997) New method for retrospective detection of exposure to organophosphorus anticholinesterases: application to alleged sarin victims of Japanese terrorists. *Toxicol. Appl. Pharmacol.* **146**, 156–161.

(14) Nagao, M., Takatori, T., Matsuda, Y., Nakajima, M., Iwase, H. and Iwadate, K. (1997) Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. *Toxicol. Appl. Pharmacol.* **144**, 193–203.

(15) Matsuda, Y., Nagao, M., Takatori, T., Niijima, H., Nakajima, M., Iwase, H., Kobayashi, M., and Iwadate, K. (1998) Detection of the sarin hydrolysis product in formalin-fixed brain tissues of victims of the Tokyo subway terrorist attack. *Toxicol. Appl. Pharmacol.* **150**, 310–320.

(16) Ott, D. G. (1986) Synthesis Of Isotopically Labeled Threat Agents. In *Synthesis and Applications of Isotopically Labeled Compounds 1985* (Muccino, R. R., Ed.) Proceedings of the Second International Symposium, Kansas City, MO, 3–6 September 1985, pp 409–414, Elsevier Science Publishers BV, Amsterdam.

(17) Lockridge, O., and La Du, B. N. (1978) Comparison of atypical and usual serum cholinesterase. Purification, number of active sites, substrate affinity and turnover number. *J. Biol. Chem.* **253**, 361–366.

(18) Wijkmans, J. C. H. M., Meeuwenoord, N. J., Bloemhoff, W., Van der Marel, G. A., and Van Boom, J. H. (1996) Solid-phase synthesis of alkylphosphonopeptides. *Tetrahedron* **52**, 2103–2112.

(19) Nachon F., Ehret-Sabatier L., Loew D., Colas C., Van Dorsselaer A., and Goeldner M., (1998) Trp82 and Tyr332 are involved in two quaternary ammonium binding domains of human butyrylcholinesterase as revealed by photoaffinity labeling with [^{3}H]DDF. *Biochemistry* **37**, 10507–10513.

(20) Lockridge, O., Bartels, C. F., Vaughan, T. A., Wong, C. K., Norton, S. E., Johnson, L. J. (1987) Complete amino acid sequence of human serum cholinesterase. *J. Biol. Chem.* **262**, 549–555.

(21) Doorn, J. A., Gage, D. A., Schall, M., Talley, T. T., Thompson, C. M., and Richardson, R. J. (2000) Inhibition of acetylcholinesterase by (1*S*,3*S*)-isomalathion proceeds with loss of thiomethyl: kinetic and mass spectral evidence for an unexpected primary leaving group. *Chem. Res. Toxicol.* **13**, 1313–1320.

(22) Doorn, J. A., Schall, M., Gage, D. A., Talley, T. T., Thompson, C. M. and Richardson, R. J. (2001) Identification of butyrylcholinesterase adducts after inhibition with isomalathion using mass spectrometry: difference in mechanism between (1R) and (1S)-stereoisomers. *Toxicol. Appl. Pharmacol.* **176**, 73–80.

(23) Elhanany, E., Ordentlich, A., Dgany, O., Kaplan D., Segall, Y., Barak, R., Velan, B., and Shafferman, A. (2001) Resolving pathways of interaction of covalent inhibitors with the active site of acetylcholinesterases: maldi-tof/MS analysis of various nerve agent phosphyl adducts. *Chem. Res. Toxicol.* **14**, 912–918.

(24) Galea, C. A., Dalrymple, B. P., Kuypers, R., and Blakeley, R. (2000) Modification of the substrate specificity of porcine pepsin for the enzymatic production of bovine hide gelatin. *Protein Sci.* **9**, 1947–1959.

(25) Powers, J. C., Harley, A. D., and Myers, D. V. (1977) Subsite specificity of porcine pepsin. *Adv. Exp. Med. Biol.* **95**, 141–157.

(26) Masson, P., Marnot, B., Lombard, J. Y., and Morelis, P. (1984) Electrophoretic study of aged butyrylcholinesterase after inhibition by soman. *Biochimie* **66**, 235–249.

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