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Retrospective detection of exposure to organophosphorus anti-cholinesterases: fluoride reactivation and mass spectrometric analysis of phosphylated human butyrylcholinesterase

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With the recent proliferation of terrorism and the constant threat of chemical warfare, rapid, sensitive and reliable methods are required for retrospective detection of exposure to chemical warfare agents. Anti-cholinesterase organophosphates react rapidly with the serine-198 residue in the active site of human butyrylcholinesterase (BuChE), under formation of a phosphate or phosphonate ester. The rather long half-life time and relatively high concentration of this enzyme renders it a persistent and abundant source for biomonitoring of exposure to organophosphate anti-cholinesterases. In recent years, our group developed a procedure for analysis of phosphylated BuChE in plasma or serum samples, which is based on reactivation of the phosphylated enzyme with fluoride ions, under formation of the corresponding phosphofluoridate that can subsequently be quantitated by GC-MS. The retrospectivity of this method was studied by the injection of a signfree dose of nerve agent in rhesus monkeys. Unfortunately, it can be envisaged that not every type of phosphyl moiety is susceptible to fluoride reactivation, *e.g.*, after aging of the inhibited enzyme in case of soman. We here present a novel and general procedure for diagnosis of exposure to organophosphates, which surpasses the limitations of the fluoride reactivation method. This method is based on the rapid isolation of BuChE from plasma by affinity chromatography, followed by mass spectrometric analysis of phosphylated nonapeptides resulting after digestion of inhibited BuChE with pepsin. We demonstrate that this method can be applied for unambiguous detection of exposures to various nerve agents, including soman.

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INTRODUCTION

The number of countries that has signed the treaty for prohibition of the production and stockpiling of Chemical Warfare Agents (CWA) is still increasing. However, the threat of use of chemical weapons has not decreased. Several examples of incidents in which CWA were involved can be given such as, the Iran-Iraq war in the 1980's, the threat of CWA use in the Gulf War, the nerve agent attack against the Kurdish population in Iraq and several terrorist assaults in Japan in the mid 90's.
It is obvious that reliable and sensitive detection methods are required to verify supposed exposures to CWA because,

- From the military-political point of view it is desirable to establish and monitor possible casualties on the battlefield.
- It is likely that a health surveillance program should be set up for employees working in CWA destruction facilities. Recently, the OPCW also addressed the issue whether biosamples taken during their inspections should be screened for CWA traces.
- In the case of a terrorist assault, unambiguous verification of CWA exposure is needed because this information can be used as forensic evidence.

Biomarkers have to meet several criteria to be an adequate marker. Firstly, the biomarker must be specific for exposure to CWA. Secondly, the method must be very sensitive, *i.e.*, low dose exposures or low concentrations of biomarker must still be detectable. Thirdly, the persistence of the biomarker should be significant which means that even at a considerable period of time after exposure, the biomarker must still be present and detectable. In order to address these issues several strategies can be followed. After exposure to the CWA, the parent compound will circulate intact for a short period of time (1, see also Figure 1). It can be attempted to measure this compound. However, the lifetime of most of the CWA is rather short and detection will not be possible for more than approximately six hours after exposure. Metabolites of CWA circulate for a longer period of time and are mostly excreted in urine. O-isopropyl methylphosphonic acid, a metabolite of sarin, could be traced in urine and plasma samples derived from victims after the assault in the Tokyo subway (2). However, the retrospectivity of this method is limited because of the polar nature of these compounds, which will be excreted rather rapidly.

We focussed on the formation of adducts to biomolecules. It is anticipated that the persistence of these molecules will be much longer than that of the metabolites. The primary consequence of a nerve agent exposure is inhibition of the enzyme acetylcholinesterase (AChE) yielding an excess of acetylcholine, which results in paralysis of the respiratory function. The concentration of butyrylcholinesterase (BuChE) in blood is higher than that of AChE. Consequently, a significant part of the nerve agent will be bound to BuChE. Therefore inhibited BuChE might serve well as a biomarker for exposure to nerve agents. Measurement of ChE activity is sensitive and rather easy but has also some disadvantages.

(i) The inter-individual variance of ChE activity is rather large implicating that an inhibition level less than 20% is not significant.
(ii) Other compounds than nerve agents, for example drugs, can influence the ChE activity.
(iii) From BuChE activity measurements the identity of the inhibitor cannot be elucidated. Evidently, BuChE activity measurements alone cannot serve as evidence in forensic cases.
(iv) The retrospectivity of ChE activity measurements is also limited, due to *de novo* synthesis. ChE activity levels are restored at approximately ten days after a single sub-lethal exposure.

Instead of measuring a decrease of ChE activity, we chose to analyze ChE that has been inhibited by organophosphates.

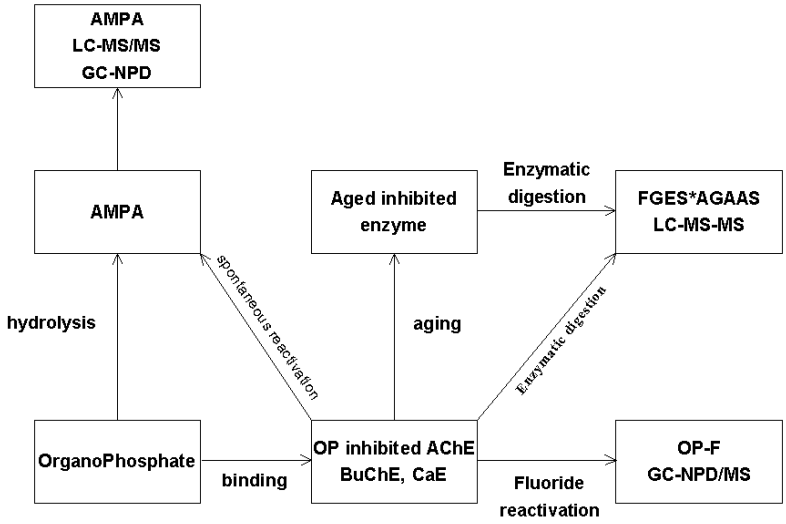

Figure 1: Fate of organophosphates and potential targets for diagnosis/verification of exposure

It was found that during incubation in the presence of a high concentration of fluoride ions, the organophosphate is released from the enzyme thus yielding a phosphofluoridate which can be analyzed with gas chromatography and NP detection or mass selective detection (3). The retrospectivity of this method was tested in rhesus monkeys which were injected with a sign free dose of nerve agent. Limitations of this method are aging and spontaneous reactivation. Spontaneous reactivation results in the formation of alkylmethylphosphonic acids. Analytical methodology is available for such acids, utilizing LC-MS (4) or GC-MS (2,5). Aging results in the formation of a methylphosphonic acid adduct conjugated to serine^{198} in BuChE. In this paper a new method is described for the isolation of BuChE from plasma followed by the digestion with pepsin and analysis with LC-MS.

EXPERIMENTAL

Two unanesthetized rhesus monkeys (*M. mulatta)*, were exposed (i.v.) to sublethal amounts of soman, sarin or VX, after written approval of the protocol by the ethical committee. Atropine sulphate (28.5 µg/kg) was administered (i.m., hind leg) 10 min before the administration of the nerve agents in the *vena saphena* of the right hind leg. Blood was sampled by vene puncture from the other hind leg. Nerve agents were dissolved in saline and administered portionwise, guided by cholinesterase activity measurements in blood after each administration, until ca. 30-40% inhibition of BuChE activity was reached. Plasma samples were incubated with acetate buffer and potassium fluoride (0.25 M) for 15 min. Next, an internal standard was added, after which the mixture was purified over a Nexus ABSelute cartridge (Varian). The generated phosphofluoridates were eluted with ethyl acetate.

Routinely, generated sarin (GB) from fluoride-treated blood samples was analyzed by means of gas chromatography with large volume sample introduction using thermodesorption cold trap (TCT) and NP detection. Regenerated soman (GD) en ethyl sarin (regenerated after inhibition with VX) were measured with GC-MS using thermodesorption. The MS was operated using chemical ionization with ammonia as ionization gas (6).

BuChE was isolated from human plasma using procainamide affinity chromatography as described by Fidder *et al* (7). Briefly plasma is applied on a column loaded with procainamide gel and eluted with a gradient of sodium chloride in phosphate buffer.

Purified HuBuChE was then incubated with pepsin in 5% aqueous formic acid for 2 h at 37 °C as described by Fidder *et al* (7). The peptides were filtrated through a 3 kD cut off filter and analyzed with LC-MS. The LC-MS instrument consisted of a QTOF instrument (Micromass, Altrincham, UK) and an Alliance type 2690 liquid chromatograph (Waters, Milford, MA, USA).

RESULTS

In vivo experiments with rhesus monkeys exposed to nerve agents

Rhesus monkeys were used for experiments to determine the *in vivo* life span of OP inhibited BuChE by means of analysis of the phosphofluoridate that was formed after fluoride induced reactivation. Recently the results were published for rhesus monkeys which were injected with tabun and cyclohexylsarin (8).

Figure 2 shows the amount of phosphofluoridate per ml plasma that could be generated at various time points after injection of sarin, soman or VX.

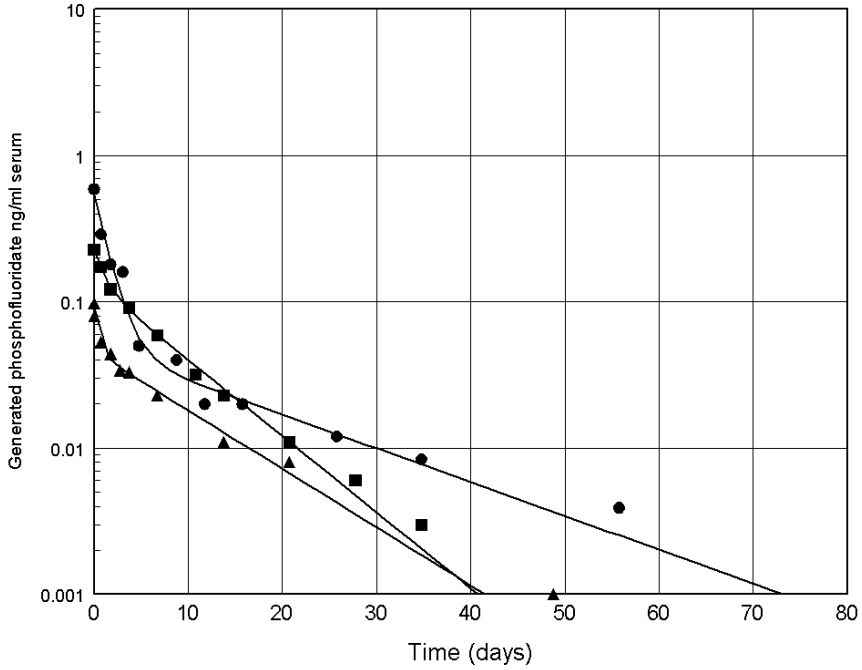

Figure 2: Amount of phosphofluoridate (ng/ml) that can be generated from rhesus monkey plasma at various time points after i.v. injection of a sign free dose of nerve agent. ▲GD, ●GB, ■ES.

The dose of administered GB was 18 nmol /kg and 5 nmol /kg (curve shown) for the two different monkeys. Both curves could be best described with a bi-exponential equation. Half-life times were 1 day and 12 days resp. GB could be regenerated from plasma until 55 days after administration. The animal experiments with VX and soman were performed in 1998 and the plasma samples were stored in the freezer at -74°C. The analytical methodology was not validated before 2001. The samples were processed and analyzed in 2001 indicating the stability of the nerve agent adducts in plasma, once stored in the freezer.

The dose of VX was 0.75 nmol/kg (curve shown) for the two monkeys. The curves for ES could be described with a bi-exponential equation. Half-life times were 0.75 and 5.5 days. Ethyl sarin could be regenerated up to 35 days after administration. The dose of administered GD was 2.22 nmol/kg in order to obtain 30% BuChE inhibition. The curve shows a bi-phasic shape characterized by a first half-life time of 1.1 days and a second half-life time of 10.6 days. Soman could be generated until 48 days after administration. This is a remarkable result because the regeneration of soman with fluoride from human plasma *(in vitro)* is not possible. Apparently, there is a difference between the primary and secondary structure of BuChE from rhesus

monkey and human, or soman can be regenerated with fluoride ions from non-specific binding sites present in plasma of the rhesus monkey. Therefore it is necessary to focus on an analytical method for the analysis of aged BuChE.

Analysis of aged BuChE

The conjugate of a nerve agent to BuChE is a potential biomarker for diagnosis of nerve agent exposure. If it is not possible to release the phosphyl moiety from the enzyme, the conjugate itself must be analyzed. We applied the following strategy: (i) isolate BuChE from plasma, (ii) digest BuChE with an appropriate enzyme, (iii) analyze the formed peptide with LC-MS. The isolation of BuChE from human plasma, in particular on a large scale, using procainamide affinity chromatography has been described by Ralston *et al* (10). We used the same technology on a small scale in order to isolate BuChE from plasma. Plasma (2 ml) was loaded onto the column filled with procainamide gel. Matrix proteins were washed away with phosphate buffer and BuChE was eluted with high ionic strength buffer (0.2 M NaCl in phosphate buffer). Figure 3 shows that native BuChE (determined with Ellman procedure) as well as inhibited BuChE (determined with radioactivity, BuChE was inhibited with ^{14}C-soman) was recovered from plasma. Figure 3 shows also that BuChE from humans and rhesus monkeys behave differently, tentatively caused by differences in structure of BuChE. Radioactivity in fraction 2 of rhesus monkey plasma is caused by free soman or its hydrolysis product. The excess of free soman in human plasma was first removed by dialysis.

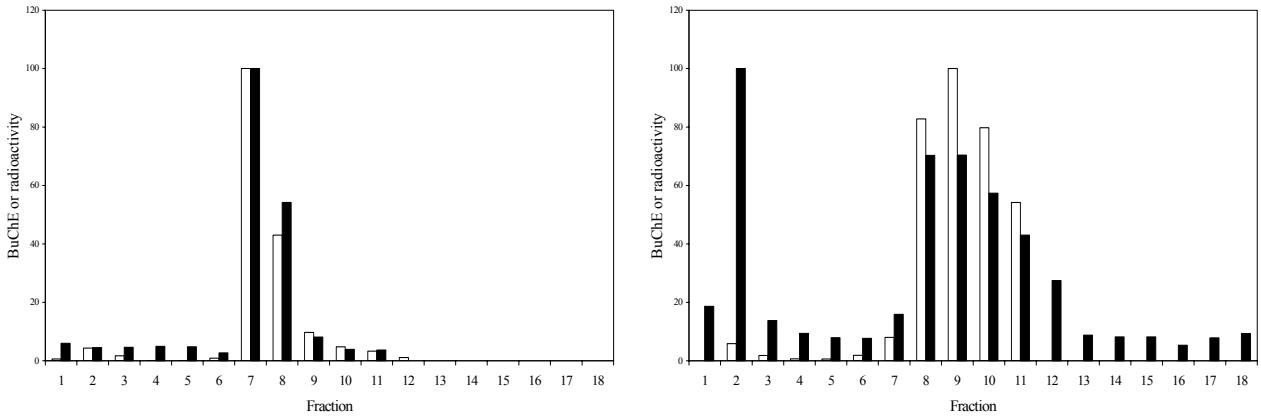

Figure 3: Elution profile of BuChE from human plasma (left) and rhesus monkey plasma (right) on a procainamide column. Open bars native BuChE activity according to Ellman, filled bars radioactivity. For conditions see Fidder et al (7).

We tried several enzymes for the digestion of purified BuChE, such as trypsin, thermolysine, pronase and finally pepsin. Pepsin appeared to be the most successful enzyme. This enzyme required a minimum of sample preparation and resulted in the formation of a nonapeptide which can be analyzed with LC-MS. The other enzymes had several disadvantages, such as requirement of additional sample preparation steps (reduction and carboxymethylation of BuChE) or the size of the formed peptide. A too large peptide is too much fragmented in the mass spectrometer which decreases the sensitivity. The disadvantages of the other enzymes are discussed by Fidder *et al* (7).

The nonapeptide, FGES(^{14}C-MPA)AGAAS, could be measured in plasma that was incubated for 30 min with different amounts of ^{14}C-soman. Figure 4 shows the mass spectrum of the nonapeptide. The protonated ion $[MH]^+$ 876.3 was selected and further fragmented. In the TOF device the ions 602.2, 673.3 and 778.3 were acquired.

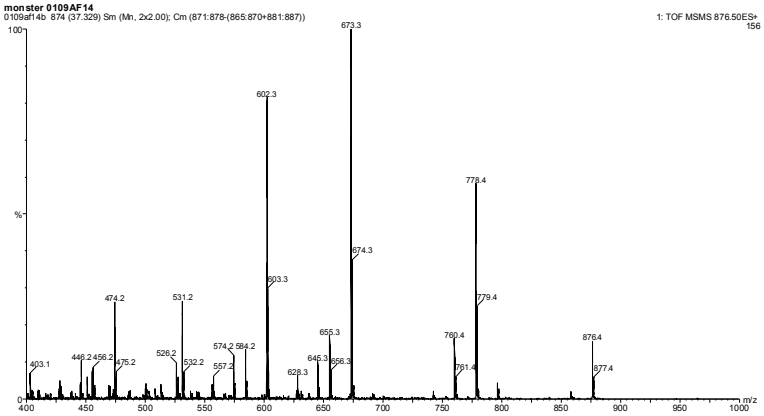

Figure 4: Mass spectrum of FGES(^{14}C-MPA)AGAAS derived after isolation of BuChE from plasma followed by digestion of BuChE with pepsin.

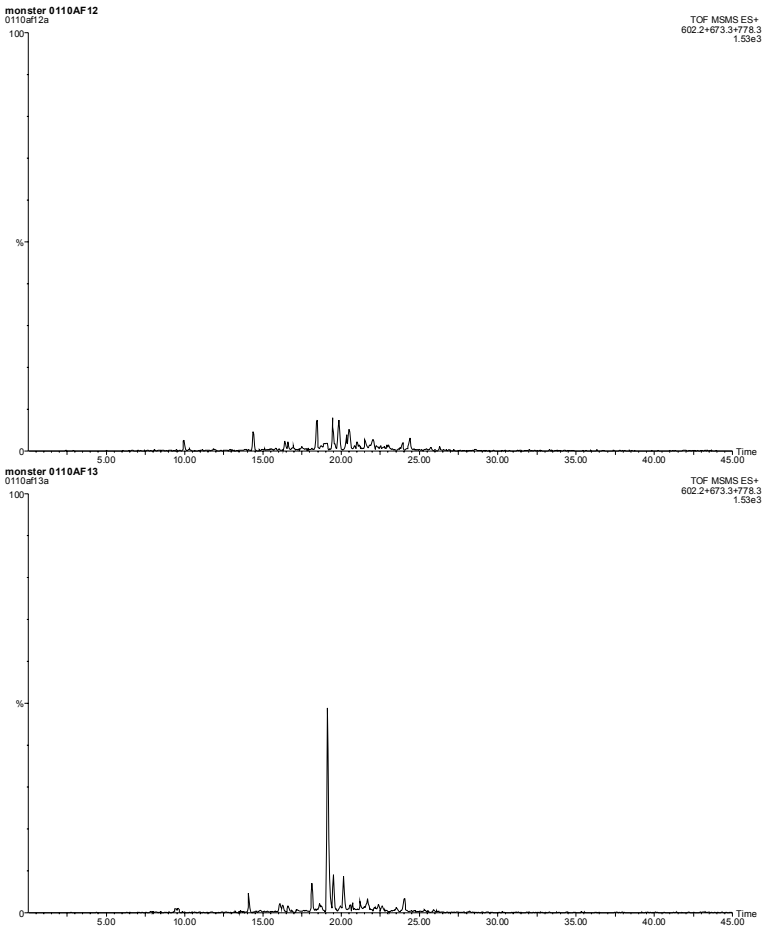

Figure 5: Summated ion chromatograms of non-inhibited BuChE (upper) and ^{14}C-soman inhibited BuChE (lower). BuChE was isolated from plasma with procainamide affinity chromatography and digested with pepsin. LC-MS: protonated ion $[MH]^+$ 876.3 was selected and fragmented ions m/z 602.2, 673.3 and 778.3 were acquired.

Figure 5 shows the summated ion chromatograms of non-inhibited BuChE and 26% inhibited BuChE.

The method is applicable for all inhibitors of BuChE, i.e. other nerve agents and pesticides, such as paraoxon. The nonapeptide conjugated with isopropyl methylphosphonic acid could be detected in the peptic digest of BuChE isolated from plasma samples derived from victims which had been exposed to sarin in the subway of Tokyo in 1995 (7) (see also Fig. 6). These samples were stored in the freezer in 1995 and analyzed with the new method in 2002. The plasma sample sizes were not more than 300-500 µl and the level of inhibition was approximately 30%, indicating the sensitivity of the method.

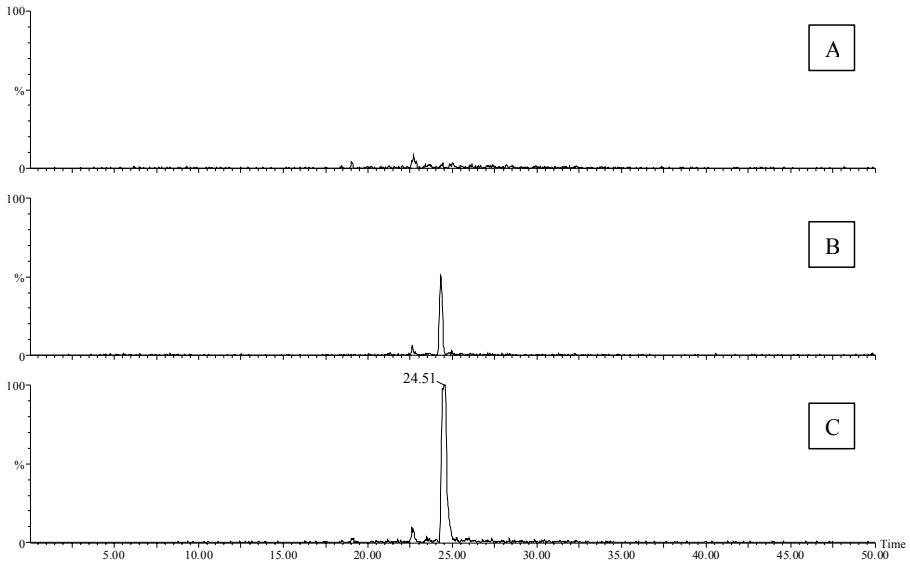

Figure 6: Ion chromatogram of m/z 778.4 after fragmentation of protonated molecular ion $[MH]^+$ 916.5, FGES(IMPA)AGAAS. (A) peptic digest of BuChE derived from non-exposed plasma, (B) peptic digest of BuChE derived from plasma from a victim of the terrorist attack in the subway of Tokyo in 1995, (C) synthetic synthesized FGES(IMPA)AGAAS.

CONCLUSIONS

- Fluoride induced reactivation of OP-inhibited BuChE is a reliable and retrospective method to establish OP exposure.
- The fluoride reactivation method is limited to compounds that can be regenerated with fluoride ions. For example, soman cannot be released from human plasma, presumably because of rapid aging. (Contrary, soman could be released from rhesus monkey plasma. Retrospectivity was more than 30 days).
- Inhibited BuChE can be analyzed after isolation of BuChE with procainamide chromatography, digestion with pepsin and analysis with LC-MS.
- The newly developed method can be applied for all inhibitors of BuChE such as nerve agents and pesticides.

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