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Analytical approaches for monitoring exposure to organophosphorus and carbamate agents through analysis of protein adducts

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Appropriate treatment of a poisoned patient requires knowing the identity of the poison. This review summarizes the methods for identifying poisoning by organophosphorus and carbamate poisons. Mass spectrometry methods identify the poison from the adducts they form with proteins in blood. The most sensitive method uses potassium fluoride to release the organophosphorus agent from its covalent binding to serine 198 of human butyrylcholinesterase. The released poison is identified by gas chromatography–mass spectrometry. The drawback of this method is that it does not detect exposure to agents such as soman, because butyrylcholinesterase adducts with these agents age to a non-reactivatable form. A method that detects both aged and non-aged organophosphylated adducts as well as carbamate adducts is one that digests butyrylcholinesterase with a protease and analyzes the modified peptide by mass spectrometry. This method does not distinguish between poisons that have the same mass after reaction with butyrylcholinesterase – for example, between exposure to chlorpyrifos oxon and paraoxon. Albumin forms a stable, covalent bond with organophosphates on tyrosine 411. The rate of reaction with albumin is much slower than with butyrylcholinesterase, but albumin adducts persist for a longer time in the circulation; they do not age; and they do not release the organophosphate when a patient is treated with an oxime. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: mass spectrometry; proteolysis; low-dose exposure; retrospective analysis; forensic analysis

Introduction

Toxicity

Organophosphorus agents and carbamates are toxic. Exposure to these compounds inhibits acetylcholinesterase resulting in the build-up of acetylcholine in the synapses of (1) the central nervous system neurons, (2) the peripheral neurons (muscarinic), and (3) the neuromuscular neurons (nicotinic). Moderate-to-high level exposure elicits a neurotoxic response that is characterized by giddiness, anxiety, headache, tremor, confusion, convulsions, rhinorrhea, bronchorrhea, sweating, salivation, miosis, abdominal cramps, diarrhea, bradycardia, muscle fasciculation, hypertension, and/or weakness.[1–3] Acute intoxication can also cause convulsions, respiratory failure, and/or cardiac arrhythmias that can lead to death. Individuals who survive acute organophosphylate intoxication can experience other conditions. There are three areas in which delayed symptoms have been defined: the intermediate syndrome, long-term neurological effects, and organophosphate-induced delayed neuropathy (OPIDN).

One to four days after moderate-to-acute organophosphylate intoxication, some individuals develop partial paralysis in their limbs. This lasts a few weeks before full recovery. The condition is referred to as the intermediate syndrome.[2]

Some survivors of acute organophosphylate intoxication show subtle long-term, neurological defects that involve intellectual functioning, academic skills, flexibility of thinking, and motor skills. They also exhibit problems with depression, irritability, confusion, and social withdrawal. Though these symptoms could be the direct result of organophosphylate reactions, they could also be caused by cerebral anoxia. The convulsions, respiratory failure, and cardiac arrhythmias associated with acute intoxication can cause cerebral anoxia. It is generally accepted that central nervous system (CNS) anoxia can be detrimental to normal, long-term brain function.[1,2] In addition, the trauma resulting from acute exposure can lead to post-traumatic stress disorder which is a long-term disability exhibiting many of these neurological symptoms. Post-traumatic stress disorder was documented for survivors of the sarin gas attack on the Tokyo subway in 1995.[2]

The third form of delayed symptomalogy, OPIDN, stems from exposure to a subset of organophosphylates that include diisopropylfluorophosphate, tri-o-cresyl phosphate, and mipafox, among others.[4,5] The initial symptoms of this condition appear 1–2 weeks after exposure and include muscular incoordination, numbness, tingling, fatigue, and/or a cramp-like pain in the calf muscles. This progresses into moderate to severe muscular weakness and paralysis in the legs. In severe cases, the upper limbs also may be affected. Recovery is slow and is seldom complete.[2]

In addition to long-term effects from high-dose exposure, toxicity from chronic, low-dose exposure to organophosphates has been described. Low-dose exposure is defined as exposure that causes no signs or symptoms of cholinergic distress. Occurrence of this condition is controversial. Some early studies linked organophosphate exposure to lower performance on neuropsychological tests, while others found no effect.[6–8] Interest in chronic, low-dose exposure has been re-invigorated recently by studies that reported correlations between (1) moderately elevated levels of urinary metabolites from organophosphates

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and an increased risk (2-fold) for attention-deficit/hyperactivity syndrome in children,[9] and (2) exposure to organophosphorus pesticides (in a farming context) and an increased risk (1.5-fold) for Alzheimer's disease.[10] Other studies have recently linked chronic low-dose organophosphate exposure to an increased risk of Parkinson's disease (1.6-fold),[11] neurologic dysfunction (2.5-fold),[12] and depression (1.5-fold).[13] Cause-and-effect relationships have not been established. It is noteworthy to mention that exposure to carbamates did not correlate with increased risk of depression.[13]

Definition of organophosphylates

Organophosphorus agents considered in this review include organophosphates [$OP(OR_1)(OR_2)X$], organophosphonates [$OP(R_1)(OR_2)X$], organophosphoramidates [$OP(OR_1)(NR_2)$], and organophosphorodiamidates [$OP(NR_1)(NR_2)$] where R_1 and R_2 can be hydrogen, alkyl, or aryl moieties and X is a reactive leaving group that can take on a wide variety of structures. As a group, these compounds are referred to by the generic term 'organophosphylates'. They are used as flame retardants, plasticizers, lubricants, pesticides, and chemical warfare nerve agents.[14] Organophosphate pesticides typically are manufactured in a phosphorothion-form where the phosphoryl oxygen is replaced by sulfur [$SP(OR_1)(OR_2)X$]. The phosphorothion-form is less toxic than the phosphoryl-form. The thion is activated by oxidation to the oxon by cytochrome P-450.[15] Carbamates considered in this review are used as pesticides.

An illustrative collection of organophosphylate and carbamate structures is given in Table 1.

Populations at risk from exposure to pesticides and nerve agents

Both organophosphate and carbamate pesticides pose a potential threat to farmers (and to other people in farm communities), to professional pesticide applicators, and to workers in pesticide production facilities.[1,3,16] Despite a decline in the use of pesticides in recent years (due to governmental proscriptions such as the Food Quality Protection Act issued by the United States in 1996), pesticides are still widely used. For example, 33 million pounds of organophosphate pesticides were used in the United States in 2007.[17] Such widespread usage makes it difficult for anyone to avoid exposure to active pesticide or its metabolites. Also at risk are first responders (police, emergency workers, and hospital staff) who assist victims of high-dose exposure to pesticides.[3] Though it is important to consider the toxicity of carbamate pesticides, the majority of the literature addresses issues with organophosphates.

Organophosphylates, in the form of nerve agents, pose a special threat to the military: including active-duty combat personnel, nerve agent stockpile handlers, and nerve agent demilitarization workers.[18,19] In addition, the general public is at risk of exposure to nerve agents due to their use for terrorist purposes.[20–23]

Some organophosphates are used in plasticizers, lubricants and flame retardants. These are phosphoric acid triesters that lack a reactive leaving group.[14] They are mixed into the products in small amounts but are not chemically bound to the material. Consequently, they are continuously released into the environment. However, since these organophosphates lack an active leaving group they are relatively non-reactive[14] and are generally considered to pose little health risk to the general public or to the workers who manufacture them. A significant exception to this generalization is tri-cresyl phosphate that is used as an anti-wear additive for jet engine oil (for more detail on tri-cresyl phosphate see the section entitled 'Proteolysis of labelled protein – Butyrylcholinesterase, phosphate').

Benefits from detection of pesticides and nerve agents

Because of the health risks posed by pesticides and nerve agents, detection of exposure has become a priority. Proper exposure assessment is important in general for determining (1) the course of medical treatment for acutely exposed individuals, (2) for preventing further contamination to first responders and hospital staff through secondary exposure,[3,18,24,25] (3) for the performance of epidemiological studies on vulnerable populations such as children[26] or farm workers,[16] and (4) to gauge the potential for development of long-term afflictions such as organophosphate-induced delayed neuropathy[1,2] or the organophosphate-induced intermediate syndrome.[1] From a military-political perspective, robust methods for detection of nerve agents are desirable (1) to assess the cause of battlefield casualties, (2) to verify adherence to the Chemical Weapons Convention, (3) as forensic tools to identify the agents involved in terrorist attacks, and (4) to provide reliable forensic evidence suitable for use in legal proceedings such as in the identification of individuals who have handled nerve agents.[18,24,27,28] In addition, because the health risks from low-level exposure to nerve agents and pesticides have not yet been fully explored, it is important to be able to assess exposure for personnel who may have been exposed but who exhibit no symptoms so that proper remedial action can be initiated if unexpected symptoms develop over time. Sensitive and reliable means for assessing low level exposure also make it possible to reassure people that they have not been exposed even though they may have been in the vicinity of a nerve agent/pesticide release.[18,24,27]

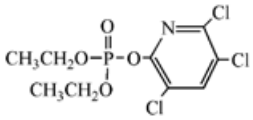
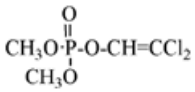
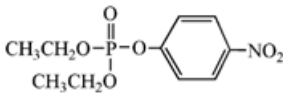
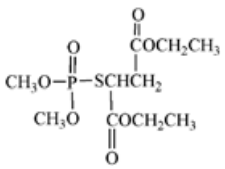
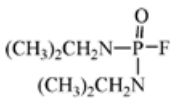
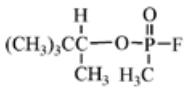
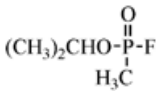
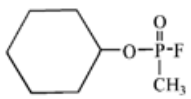
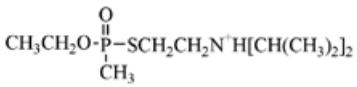
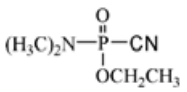
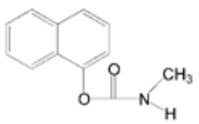
Assays for exposure to organophosphylates

Three types of assay have been developed for determining exposure to organophosphylates and carbamates: (1) monitoring the activity of acetylcholinesterase or butyrylcholinesterase for inhibition, (2) direct detection of the toxicants or their metabolites (in serum, saliva, urine and the environment), and (3) detection of in vivo reaction products that arise from these compounds (protein adducts). Thorough reviews are available on detection of exposure through the use of cholinesterase activity[24,29–31] and through direct detection of organophosphylates, carbamates and their metabolites.[14,18,19,24,32–34] Only limited reference to these topics will be made in this presentation. The primary focus will be on detection of protein-adducts as biomarkers for exposure.

Monitoring of cholinesterase activity

The earliest assays for assessing exposure to organophosphylates and carbamates used inhibition of butyrylcholinesterase or acetylcholinesterase activity.[18] Carbamate and organophosphylate inhibitors of cholinesterases work by promoting a partial substrate reaction.[29,30,35] Normal substrate hydrolysis is a two-step process involving rapid alkylation of the active-site serine followed by rapid hydrolysis of this enzyme-substrate complex to reform the starting enzyme[30] (Figure 1).

Table 1. Selected list of toxicants.

Common name[a]	Chemical name CAS number	Structure
Organophosphate Pesticides		
Chlorpyrifos oxon	O,O-diethyl-(3,5,6-trichloro-2-pyridyl) phosphate CAS 2921-88-2	
Dichlorvos	O,O-dimethyl-(2,2-dichloroethenyl) phosphate CAS 62-73-7	
Paraoxon	O,O-diethyl-(4-nitrophenyl) phosphate CAS 311-45-5	
Malaoxon	O,O-dimethyl- S-(1,2-dicarboethoxy-ethyl) phosphorothioate CAS 1634-78-2	
Mipafox	N, N′-diisopropyl phosphorodiamidofluoridate CAS 371-86-8	
Organophosphylate Chemical Warfare Agents		
Soman (GD)	O-(3,3-dimethylbutyl) methylphosphonofluoridate CAS 96-64-0	
Sarin (GB)	O-isopropyl methylphosphonofluoridate CAS 107-44-8	
Cyclosarin (GF)	O-cyclohexyl methylphosphonofluoridate CAS 329-99-7	
VX	O-ethyl-S-[2-(diisopropylamino) ethyl] methylphosphonothiolate CAS 50782-69-9	
Tabun (GA)	O-ethyl-dimethylamino phosphoramidocyanate CAS 77-81-6	
Carbamate Pesticides		
Carbaryl	1-naphthyl-N methylcarbamate CAS 63-25-2	

[a]The abbreviations GA, GB, etc refer to members of the G-series of nerve agents, so called because they were first synthesized by German chemists during World War II.

The VX abbreviation designates members of the V-series of nerve agents, a second generation. Depending on the source, the V stands for Victory, Venomous, or Viscous.

Inhibition begins similarly, with rapid phosphylation/carbamylation of the active-site serine to form a covalent enzyme-inhibitor complex. However, the reaction is arrested at this point by slow hydrolysis.[30,35] The process is illustrated in Figure 2 using an organophosphate inhibitor.

Rates for spontaneous hydrolysis of the enzyme-inhibitor complex are orders of magnitude slower than those for the normal substrate reaction.[30] Thus, formation of the phospho-enzyme complex effectively inhibits activity. Slow hydrolysis for carbamate-enzyme complexes can be attributed to the greater stability of the amide linkage relative to the ester. Slow hydrolysis for organophosphylate-enzyme complexes has been attributed to the positioning of the phosphorus adduct which prevents it from taking advantage of the normal enzyme machinery to catalyze hydrolysis.[35]

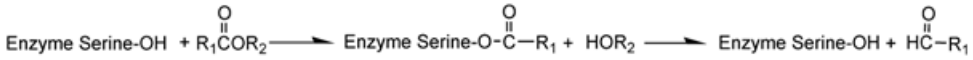


Figure 1. Substrate turnover with serine esterases.

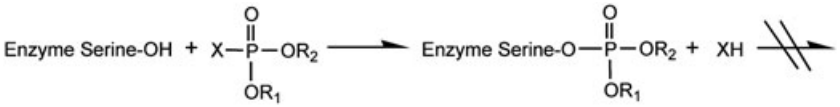


Figure 2. Organophosphate inhibition of serine esterases.

Cholinesterase activity measurements are simple, rapid, convenient and cheap.[16,24,31,36] They typically are made on blood (serum, plasma, red blood cells, or whole blood). Obtaining samples of blood is minimally invasive.[31] Unfortunately, determination of exposure by measuring butyrylcholinesterase or acetylcholinesterase activities is compromised by a relatively large individual-to-individual variation in normal activities.[29] For butyrylcholinesterase the difference between low and high activities has been reported to vary from 2.4–3.5-fold. Data in support of this statement come from two separate studies. In the first study, measured activities varied from 1.35–3.23 μmoles of butyrylthiocholine/min/ml of whole blood.[28] In the second, activities varied from 2.5–8.7 μmoles of acetylthiocholine/min/ml of serum.[16] For acetylcholinesterase the reports of differences vary from 1.6–2.3-fold. Again, data are from two separate studies. In the first, activities varied from 0.6–0.98 delta pH/hour from hydrolysis of acetylcholine.[36] In the second, activities varied from 6.0–14.0 μmoles of acetylthiocholine/min/ml of blood.[16] The exact cause for variability can be complicated. Gender, age, and health status contribute,[37] as do genetic variants.[26] Levels of activity can be affected by pregnancy, medications and/or illegal drugs,[26,38] as well as by alcoholism, x-ray therapy, acute infection, malnutrition and diseases such as hepatocarcinoma, hepatitis, asthma, hypertension, and tuberculosis, among others.[38] In order to accurately assess exposure to inhibitors, especially for low-level exposures that do not cause cholinergic symptoms, an un-inhibited reference activity is needed from each individual. Even then, activity assays do not provide reliable evidence for exposure when the inhibition levels are less than 20%.[18,39] For butyrylcholinesterase (assuming an average level in human plasma of 50 nM,[40] 20% inhibition would mean that the lower limit of detection for toxicants is about 10 nM. For acetylcholinesterase (assuming an average level in human red cells of 8 nM,[41] 20% inhibition would mean that the lower limit of detection is about 2 nM. In addition, activity assays cannot identify the type of inhibitor to which an individual was exposed.[18,39] It cannot even discriminate between organophosphylate and carbamate inhibition. Finally, activity measurements are not amenable to retrospective analysis because new enzyme replaces the inactive enzyme relatively rapidly.[18,39]

Despite its limitations, cholinesterase activity provides a simple, semi-quantitative measure of exposure to organophosphylates and carbamates. It is the primary method used by the US Army for routine evaluation (Walter Reed Army Institute of Research Whole Blood cholinesterase assay[31] or the Michel delta pH assay[36]), and is employed for conventional epidemiological studies.[16] A rapid, field-deployable monitor based on electrochemical determination of cholinesterase activity has been developed[42] as well as a portable absorbance-based detector[43] (Test-mate ChE™). Applications for high-throughput are also available.[24] Determination of red cell acetylcholinesterase activity, mirrors the activity of acetylcholinesterase in peripheral synapses[29] and is valuable for deciding on the duration of antidotal oxime therapy in cases of rapidly aging or long persisting agents.[24] Nevertheless, the limitations implicit in the activity assays fostered the development of other methods to assess exposure to organophosphylates and carbamates.

Direct detection of organophosphylates/carbamates and their metabolites

Early methods for direct detection of the toxicants employed gas chromatography (GC) and a variety of schemes for monitoring the effluent. However, since the introduction of electrospray ionization mass spectrometry (ESI-MS) in the 1990s, liquid chromatography with mass spectrometric (LC-MS) detection has come to dominate the field.[32] The source of the samples for direct detection is usually urine or serum (plasma), although saliva, amniotic fluid and meconium (faeces from new born) have been used.[34] Partial purification of the analyte is generally required and involves three to four steps:

(1) Isolation/enrichment of the analyte is most often accomplished by liquid-liquid extraction (LLE) or solid-phase extraction (SPE).
(2) When GC is the separation technique, derivatization is required. This step is unnecessary when LC is used for separation.
(3) Separation of the analytes is generally performed by GC or LC. However methods involving ion mobility[44] and capillary electrophoresis[19] have also been described.
(4) When GC is used, detection of the analytes is accomplished by flame photometry, flame ionization, nitrogen-phosphorus detection, or MS. Simple MS or tandem mass spectrometry (MS/MS) is used for detection with LC.[14,19,32–34] MS, because of its ability to add mass information to the identification and because of its sensitivity, is becoming the detection method of choice.

The majority of methods described in the literature refer to detection of metabolites because the original toxicant is rapidly degraded *in vivo* (within a few hours to days).[14,24,32] Limits of detection (LOD) for flame ionization detection, nitrogen phosphorus detection, or flame photometric detection are in the mid-to-upper μg/L (urine or blood) range.[33] Using simple mass spectral detection, the limit is generally in the low-μg/L range, while tandem mass spectral methods are frequently able to detect exposure at sub-μg/L.[33,34] One μg/L translates into about 5 nM (assuming an analyte molecular weight of about 200 gm/mole). The preferred source of sample for metabolites

is urine. Methods that employ serum (plasma) are generally used for forensic purposes or for acute intoxication. LOD from serum are in the μg/L to mg/L range.[33]

GC methods for measuring the toxicant directly or its metabolites resolved the issue of individual-to-individual variation that plagued the cholinesterase activity method, and they improved on the sensitivity of detection. However, they are limited to analytes that are volatile, of low polarity, and thermally stable. In addition, GC requires that the analyte be derivatized and be delivered in organic solvent which complicates sample preparation.[14,33,34] These drawbacks were overcome by the introduction of LC methods. LC is compatible with a wider variety of samples, does not require derivatization, and aqueous samples can be used directly without sacrifice of sensitivity.[14,32–34]

Use of mass spectral detection also improved identification of the toxicant. For example, the mass information allows carbamate toxicants to be differentiated from organophosphylate toxicants. However, most methods still rely on metabolite analysis.[14,32] Metabolic degradation of an organophosphylate typically involves hydrolysis of the reactive group leaving stable phosphorus metabolites that are common to a number of toxicants. For example, the organophosphates paraoxon, chlorpyrifos oxon, and diazoxon all yield diethylphosphate. Dialkyl phosphates are the most commonly monitored analytes of exposure to organophosphorus pesticides and nerve agents. Monitoring of the reactive leaving group would improve the identification process, but it is generally more labile than the phosphorus metabolite and is therefore less amenable to detection. Detection of the reactive metabolite as well as the phosphorus metabolite can be made for selected toxicants, such as trichloropyridinol for chlorpyrifos oxon,[33,34] but in general the original toxicant cannot be precisely identified.[24,33,34]

Metabolite analysis also suffers from an inability to determine the differences between metabolites that were generated in vivo or generated in the environment and consumed as such by the subject.[26]

Tandem mass spectral analysis further improved the quality, selectivity and sensitivity of identification for both metabolites and the parent toxicants. Quality was improved through fragmentation analysis which provided a more confident identification than relying on parent ion mass alone. Selectivity and sensitivity were improved through the use of single (or multiple) reaction monitoring wherein a precursor/fragment ion-pair is monitored, thereby reducing background interference.[32] However, MS/MS could not overcome the lack of specificity engendered by targeting metabolites rather than intact toxicants.

Another drawback for direct analysis of toxicants and their metabolites is their short lifetime *in vivo*. Urinary metabolites are generally gone within 2–3 days,[18,20,24,32] though they may be detectable for up to two weeks depending on the toxicant, the dose received and the sensitivity of the analytical method.[45] This short life-time severely limits retrospective analyses.

Detection of *in vivo* reaction products (protein biomarkers)

Analysis of protein biomarkers was adopted to improve retrospective detection of exposure to organophosphylates.[24] Proteins that have been used for detection of exposure to organophosphylates are butyrylcholinesterase,[22,40,46–48] acetylcholinesterase,[21,49,50] and serum albumin.[45,51] For exposure to carbamates, only butyrylcholinesterase has been described.[40,48,52] Discussions on the use of protein biomarkers have been included in several reviews.[14,18,24,53] Ideally, a biomarker should be present in an easily obtainable matrix (such as blood, urine, or saliva), should be relatively long-lived, should provide unequivocal identification of the toxicant, and should be detectable at low concentrations.[28,45] To date no method for detection of organophosphylate/carbamate biomarkers fulfills all of these requirements. Methods available for analysis of protein biomarkers can be divided into two categories: (1) dissociation of the toxicant from the protein-adduct followed by detection of the toxicant and (2) proteolysis of the labelled protein followed by detection of the released peptide-adduct.

Dissociation of toxicant from protein-adducts

The first reports on the use of protein biomarkers to assess exposure to organophosphylates were made in 1997, in the wake of the sarin terrorist attack on Tokyo, Japan.[21,22] Two laboratories developed analytical methods. Investigators in both laboratories dissociated the organophosphonate-adduct from the inhibited enzymes and measured the released product. Polhuijs and coworkers used fluoride to dissociate the inhibitor from butyrylcholinesterase in serum.[22] Nagao *et al*. isolated inhibited acetylcholinesterase from red cells, trypsinized it, and released the inhibitor from the tryptic peptide with alkaline phosphatase.[21]

Dissociation of the toxicant – Fluoride reactivation

The use of fluoride to reactivate organophosphylate-inhibited cholinesterases was pioneered by Heilbronn in studies on sarin inhibited human blood cholinesterases.[54,55] Fluoride reacts with the organophosphorus-adduct to form a phosphofluoridate, returning the enzyme to its active form. The mechanism is illustrated in Figure 3 with an organophosphonate adduct. Heilbronn monitored the breakdown of the phosphorus adduct by return of enzymatic activity. She demonstrated that the rate of the reactivation was dependent on sodium fluoride concentration and that the rate increased as the pH was decreased (pH 9 to 5)[54].

Aging of the phosphorus adduct prevents fluoride reactivation.[54,56] Aging is an enzyme-mediated dealkylation of the enzyme-adduct which is particularly prevalent with cholinesterases[57,58] though it also occurs with other members of the serine-hydrolase family, for example neurotoxic esterase.[56] The mechanism is illustrated in Figure 4 with an organophosphonate adduct. A functional definition of aging is that it creates an

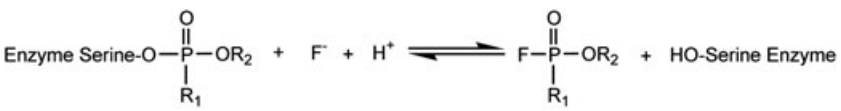


Figure 3. Fluoride reactivation.

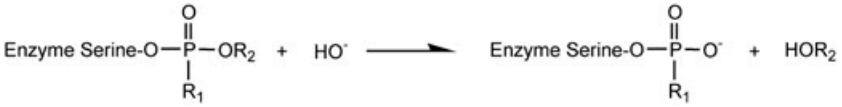


Figure 4. Aging.

enzyme-adduct complex that will not spontaneously reactivate and cannot be induced to reactivate by compounds such as oximes or fluoride.

Scattered applications of the fluoride reactivation (or regeneration) method appeared over the next 30 years. For example, Clothier and Johnson showed that KF could reactivate diisopropylfluorophosphate-inhibited hen neurotoxic esterase;[56] de Jong and van Dijk applied the method to soman-inhibited aliesterase from rats;[59] and Milatovic and Johnson used it to assess aging of neurotoxic esterase and acetylcholinesterase from hen brain after inhibition by mipafox.[60] But, it was not until Polhuijs combined potassium fluoride reactivation with MS in a retrospective study to identify the organophosphorus agent used in the 1995 nerve gas attack on the Tokyo subway that interest in the method blossomed.[22]

Polhuijs used C18-SPE (eluting with ethyl acetate) to isolate the phosphorus compound released from serum by incubation with 2 M potassium fluoride for 2 min at 25 °C and pH 4. This was followed by gas chromatography coupled with alkali-flame nitrogen-phosphorus detection to identify the released agent as sarin.[22] Improvements have been made to Polhuijs' method by several groups that have expanded its usefulness for detecting nerve agents.

Jakubowski *et al*. used sarin-treated whole blood from human, minipig, guinea pig and rat to develop their improvements. They added a large volume injector to the gas chromatograph, added a heavy-atom labelled reference ion, and replaced flame detection with mass spectral detection using ammonia chemical-ionization and single ion monitoring for the measurement of sarin.[25] Mass spectral detection yielded the mass of the released toxicant, improving confidence in its identification. In vitro, the linear range of quantitation for their method was 10–1000 pg of agent applied to the gas chromatograph, and the detection limit was approximately 2 pg. A similar study by this group using VX yielded a somewhat higher detection limit (10.5 pg).[61]

Adams *et al*. used soman- and sarin-treated human plasma for *in vitro* method development and then used treated guinea pigs and rhesus monkeys to demonstrate the practicality of their methods. They used 0.7 M potassium fluoride, extended the fluoride reaction time to 2 h and raised the reaction temperature to 40 °C to release the toxicants, then used C18 SPE followed by selected ion monitoring MS with electron ionization coupled to their gas chromatograph to detect the products.[62] Both serum and red cells were analyzed. *In vitro*, the limit of detection was 0.5 pg of product applied to the chromatograph. Both soman and sarin adducts were detected.

Detection of soman was particularly novel because fluoride is not expected to reactivate soman-inhibited human butyrylcholinesterase. Soman-inhibited human butyrylcholinesterase ages rapidly (half-life of 9 min[63]) and aged adducts cannot be reactivated by fluoride. Re-appearance of soman after reaction of soman-treated serum with fluoride (the leaving group for soman is fluoride so that fluoride reactivation regenerates the original toxicant) required extended incubation, at elevated temperature (40 °C). Using pure human albumin treated with soman, the authors showed that fluoride would release soman from albumin under these conditions; therefore they proposed that fluoride was releasing soman from albumin-adducts and not from butyrylcholinesterase adducts.

In vivo experiments with monkeys involved injection of 15 μg/kg soman (2 x LD_{50}, intramuscularly). Monkeys were treated with pyridostigmine before injection and then treated with atropine, pralidoxime and midazolam after injection. Fluoride released soman could be detected in the serum three days after injection, and in the red cells seven days after injection.[62] A variety of *in vivo* experiments were performed on guinea pigs. One involved five daily subcutaneous injections of 0.1–0.4 LD_{50} amounts of soman or sarin, followed by sacrificing the animals (1 h after the last injection) and analyzing serum, red cells, and tissues for fluoride-releasable products. Sarin could be detected in serum 2 h after sacrificing the animals, but the levels had dropped to one-half by 4 h after sacrifice. Another experiment involved 15 daily injections. Soman could be detected in the kidney 4 h after sacrifice.[62]

Degenhardt *et al*. used human plasma treated with sarin, cyclosarin, VX, or tabun for *in vitro* method development. They replaced the C18-SPE material with a Nexus (Varian Inc. Palo Alto, CA) methacrylate-divinylbenzene-based material to produce cleaner extractions. They used Tenax TA (Alltech Dearfield IL) to concentrate up to 500 μl of the solid-phase extract for introduction into the gas chromatograph. They monitored the chromatographic output by either high resolution MS with electron ionization or selected ion monitoring MS with chemical-ionization, using ammonia.[28] With these changes they achieved limits of detection in serum of 2.4 pg/ml for VX, 1.2 pg/ml for sarin, 6.3 pg/ml for tabun, and 8.6 pg/ml for cyclosarin. These levels of organophosphylate correspond to amounts needed to inhibit butyrycholinesterase by 0.02%, 0.01%, 0.05%, and 0.06%, respectively.

Holland *et al*. used human serum treated with tabun, sarin, soman, cyclosarin, and VX for *in vitro* method development. They 'made additional modifications to the re-fluoridation analysis to provide a fully validated method with improved selectivity, decreased background, an expanded linear range, and elimination of the time-consuming high-volume injection, while improving sensitivity'.[64] They adopted the Adams 1 h fluoride incubation time at 40 °C in order to add soman to the list of detectable nerve agents. They substituted chloroform for ethyl acetate in the elution step of the SPE so that the product could be readily concentrated by evaporation. They added confirmation ions to the high resolution mass spectral analysis in order to increase the selectivity of the method. In addition, they replaced the high-volume Tenax injection step with a small volume injection of concentrated product to reduce background noise. 'This modified method for nerve agent-protein adducts has lower limits of detection, greater selectivity and now includes GD [soman].'[64] The Holland method is capable of detecting (lowest reportable value) 5.5 ng/L VX (20 pM), 5.5 ng/L sarin (40 pM), 5.5 ng/L tabun (34 pM), 16.5 ng/L soman (90 pM), and 5.5 ng/L cyclosarin (30 pM). The authors assumed that there is about 80 nM butyrylcholinesterase in human serum and that essentially all of the fluoride reactivated organophosphorus agent

in serum comes from inhibited butyrylcholinesterase (with the exception of soman-inhibited butyrylcholinesterase). With these assumptions, the fluoride reactivation method is capable of detecting exposure to amounts of organophosphylate capable of inhibiting just 0.02–0.04% of the serum butyrylcholinesterase. In our experience, a more accurate number for the butyrylcholinesterase concentration in human serum is 50 nM[40,41] which would raise the detection limits to 0.04–08% of the serum butyrylcholinesterase. This level of sensitivity requires the mass spectral analysis using multiple reaction monitoring. The weakness of this approach is that prior knowledge of the nature of the toxicant is required in order to assign the parent and daughter ion masses.

Using these procedures (or minor variations on these procedures) other authors have provided further insight into the action of organophosphylates on various animals.

Byers *et al*. demonstrated that fluoride regeneration was capable of detecting whole-body exposure of Gottingen minipigs to VX vapor (10–180 min at 0.089–1.10 mg/m^3).[65] The authors concluded that fluoride regeneration from serum 'was a more precise indicator for severity of exposure than the analysis of acetylcholinesterase inhibition'.

van der Schans *et al*. treated rhesus monkeys with sufficiently small amounts of five organophosphylates (tabun, sarin, cyclosarin, VX, soman, and paraoxon) that no clinical signs were observed and butyrylcholinesterase in blood was inhibited only 30–40%.[39] They then incubated serum from these monkeys with 2 M fluoride for 15 min at 25 °C and pH 4 to release the toxicants. Products were found for all toxicants including soman. *In vitro* control experiments using these conditions with soman-treated human serum yielded no product. Under the van der Schans conditions, decreasing amounts of products from treated monkey serum could be detected for up to 50 days (7 weeks). The decrease followed a biphasic time course. The authors suggested that the 'slow phase. . .may be due to occupation of reactivatable binding sites other than BuChE', but they avoided the temptation to assign those sites to albumin.

In an earlier study, Benschop *et al*. were able to detect fluoride-released products in the blood of rhesus monkeys for 3–5 weeks after they had been intravenously injected with 1–4 μg/kg of sarin or tabun.[66]

A group of authors studied the effects of low-level, whole-body exposure of rats to vapors of soman, VX and cyclosarin.[67–71] Their goals were (1) to detect low-dose exposure, (2) to demonstrate a linear relationship between levels of exposure and levels of fluoride regenerated products, (3) to test whether fluoride regeneration would be a more sensitive assay for low-dose exposure than cholinesterase activity assays, (4) to determine the effect of low-dose exposure on previously learned tasks, and (5) to determine the effects of low-dose exposure on the ability to learn new tasks. They concluded (1) that there was a linear relationship between levels of exposure and levels of fluoride regenerated products for cyclosarin and soman but not for VX, (2) that fluoride regeneration was a more sensitive method for detection of low dose exposure than cholinesterase activity assays, and (3) that low dose exposure had no significant effect on performance of previously learned tasks or on the ability to learn new tasks.

Analysis of blood from a person accidentally exposed to VX was the second reported use of fluoride reactivation to assess in vivo human exposure to nerve agents. Results were reported by Solano *et al*.[46] and by McGuire *et al*.[49] Solano worked with serum using a slight modification of the Adams method (0.25 M potassium fluoride vs 0.7 M). McGuire worked with the red cells. Both groups were able to detect VX for up to 27 days after exposure, even though the victim was treated with pralidoxime immediately after exposure.

In summary, use of fluoride to dissociate toxicants from protein-adducts increases the time for retrospective analysis compared to direct detection of toxicant/metabolite (from a few days to 50 days). In addition, fluoride reactivation increases the sensitivity of detection compared to direct detection (from about 1 nM to 20 pM). Analyses are generally performed on serum or red blood cells. Fluoride regeneration from primate serum reflects release of toxicant from butyrylcholinesterase[39] and from albumin if the incubations conditions are stringent.[62] Regeneration from rodent serum (guinea pig, mouse and rat) reflects release from carboxylesterase, aka aliesterase.[39,59] Regeneration from red cells reflects release of toxicant from acetylcholinesterase.[25] The most significant short-coming of the fluoride regeneration method is its inability to regenerate enzyme-adducts which have undergone aging.

Dissociation of the toxicant – trypsin/alkaline phosphatase

Nagao *et al*. developed an alternative to the Polhuijs method for the detection of organophosphylates in human blood samples.[21,72] They (1) isolated acetylcholinesterase from red cells, (2) digested it with trypsin, (3) released the phosphorus-adduct with alkaline phosphatase, (4) subjected the phosphatase products to trimethylsilyl derivatization, and (5) detected the resulting compound by GC-MS using electron ionization. They used this method for retrospective detection of sarin in the blood from four victims of the 1995 gas attack on the Tokyo subway. All four victims had died from high-dose sarin exposure. A retrospective timeframe was not determined, but one might predict that it would be similar to that for turnover of the human red cell in circulation which is about 100 days. If so, this would be the most stable adduct investigated. The authors were able to detect both the isopropyl methylphosphonic acid adduct and its aged counterpart, methylphosphonic acid in all four samples even though two of the victims had been treated with pralidoxime. A similar procedure was used successfully on formalin fixed brain tissue from the same individuals.[50] No attempt was made to determine a lower limit of detection. The Nagao procedure is effective but it is substantially more labor intensive than the fluoride regeneration method. The advantage of Nagao's method over that of Polhuijs is in its ability to detect the aged adduct. However, simpler procedures have since been developed that can detect the aged adduct, which may explain why no other reports on the use of the Nagao procedure could be found. It is noteworthy to mention that adducts which appear to have been aged, can be the result of hydrolysis during digestion or during other sample preparation steps. Finally, a disadvantage of the Nagao method is that it used electron ionization which gives high chemical noise in chromatograms.

Proteolysis of labelled protein

Five years after Polhuijs used fluoride regeneration of inhibited butyrylcholinesterase to assess exposure to sarin,[22] Fidder *et al*. described a procedure for detection of organophosphylated-peptides from inhibited human butyrylcholinesterase.[47] The method involves release of the organophosphylated-peptide by proteolysis, followed by mass spectral analysis of the digest to

identify the toxicant. The concept has been applied by various groups to labelled butyrylcholinesterase, acetylcholinesterase and albumin, using a variety of proteases (trypsin, chymotrypsin, pepsin, pronase, protease type XIV, and Glu-C). It is also the basis for methods developed to detect carbamate exposure. Using this approach, it is possible to identify aged organophosphylate agents, a property lacking in the fluoride regeneration method.

Proteolysis of labelled protein – butyrylcholinesterase, organophosphylates

Fidder *et al*. used pure human butyrylcholinesterase inhibited with soman, sarin, paraoxon, or methylparaoxon for method development.[47] Four proteases were tested: trypsin, thermolysin, pronase, and pepsin. Pepsin (freshly prepared and used in 1.5-fold excess over butyrylcholinesterase, *w/w*) was ultimately selected. It was incubated with butyrylcholinesterase for 2 h at 37 °C in 5% formic acid. Reduction and alkylation prior to digestion were not necessary. These conditions were chosen because they yielded an organophosphylated nonapeptide (FGEXAGAAS where X is organophosphylated serine) that fragmented well in the mass spectrometer under collision induced dissociation conditions. The principal fragmentation was a neutral loss of the organophosphorus moiety plus a molecule of water. This yielded a major fragment at 778 amu for all organophosphylated peptides. The mass difference from the parent ion correlated with the organophosphylate used in the inhibition. Both aged and non-aged adducts could be detected. For application to serum samples, the authors performed a one-step, procainamide-affinity purification of the butyrylcholinesterase. The method was shown to have sufficient sensitivity to identify sarin as the toxicant in samples of blood taken from a victim of the 1995 gas attack on the Tokyo subway. Read *et al*. confirmed the methodology using serum from marmosets treated with sarin, cyclosarin, soman, or tabun.[45] A significant short-coming of this approach is that it relies on prior knowledge of the identity of the toxicant in order to select the mass to be fragmented.[73] This complicates the screening of large numbers of samples for unknown agents.

Noort *et al*. proposed a generic screening procedure for detecting the occurrence of organophosphylation that did not require prior knowledge of the organophosphylate.[73] They prepared the peptic nonapeptide of butyrylcholinesterase as described by Fidder. Then a portion of the digest was used for base-catalyzed beta-elimination/Michael addition (with 2-[3-aminopropylamino] ethanol) to convert the organophosphylated-nonapeptide, FGEXAGAAS (where X is organophosphylated-Ser), into FGEX'AGAAS (where X' is [$HOCH_2CH_2NHCH_2CH_2CH_2NH$]-Ser). Plasma samples exposed to sarin, soman, VX or dichlorvos all yielded the same FGEX'AGAAS peptide which appeared as a unique, diastereomeric pair of peaks in an LC/MSMS product ion chromatogram (product mass = 791 *m/z*). This method works for both organophosphorus pesticides and nerve agents. Screening samples in this way identifies those which were exposed to organophosphylates. The authors recommend that after detecting an organophosphylated sample, the original peptic digest should be re-analyzed to identify the specific organophosphylate involved.

van der Schans *et al*. proposed an alternate strategy to circumvent the necessity of knowing the identity of the organophosphylate before analysis.[27] Their strategy employs the strong 778 amu daughter ion that is generated by collision induced dissociation of the peptic nonapeptides from all forms of organophosphylated butyrylcholinesterase, and the realization that the roughly 1000 known, schedule 1, chemical warfare nerve agents can be condensed into 34 categories of parent ion. Consequently, butyrylcholinesterase (peptic peptides prepared as described by Fidder) can be screened by multiple reaction monitoring MS, using the 778 amu daughter ion together with each of the 34 parent ion masses. Because the sensitivity of multiple reaction monitoring decreases as the number of parent ions increases, the authors recommend that analysis be made using four sets of nine parent ion/daughter ion transitions. The authors described the low LOD as 10% of control values, which can be interpreted to mean 10% of the total butyrylcholinesterase in serum. Finding a positive sample defines the parent ion mass. The digest can be re-analyzed with additional characteristic daughter ions (673 and 602 amu) and/or by complete MSMS fragmentation to better define the identification. The authors did not explicitly include organophosphylated pesticides in their screening, but they did note that some of the parent ion masses for the chemical warfare agents matched pesticide masses. Regardless, the concept could easily be extended to include all organophosphylated pesticides.

Carol-Visser *et al*. augmented the Fidder method by adding on-line peptic digestion (using a Poroszyme immobilized pepsin cartridge from Applied Biosystems) for rapid determination of chemical warfare agents.[74] The lower limit of detection was equivalent to 10% inhibition of butyrylcholinesterase, if 1 ml of blood was processed.

Sporty *et al*. augmented the Fidder method somewhat differently. They added anti-butyrylcholinesterase coated ferromagnetic beads to automate butyrylcholinesterase purification thereby making the purification step amenable to high-throughput analysis.[37] They also analyzed the sample for both adducted and non-adducted butyrylcholinesterase, and added an isotopically-labelled internal standard (corresponding to the mass of the expected peptide adduct) along with an external calibration curve to increase quantitation accuracy. Analysis of both adducted and non-adducted butyrylcholinesterase provided an accurate way to determine the fraction of adducted enzyme in the sample. The low limit of detection was equivalent to 5% labelled butyrycholinesterase.

The majority of studies on the liberation of the organophosphylated peptides by proteolysis of butyrylcholinesterase were performed with pepsin. However the concept was also successfully developed using other proteases. For example, Sun and Lynn used trypsin to release the active site peptide from pure, horse butyrylcholinesterase that had been treated with paraoxon, methyl paraoxon or EPN oxon.[75] They used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (TOF-MS) to analyze the digest and were able to detect labelling at levels equivalent to 3% inhibition of butyrylcholinesterase. Both the adducted and non-adducted peptides could be analyzed in the same spectrum, providing a measure of the fraction of butyrylcholinesterase that was inhibited.

A tryptic method has also been described, to analyze blood from victims of pesticide poisoning (dichlorvos or chlorpyrifos oxon).[48] Because of the complexity of the starting material, the authors partially purified the butyrylcholinesterase from 2 ml of serum before tryptic digestion. They used a combination of ion exchange chromatography (Q-Sepharose at pH 4) and procainamide affinity chromatography. The active-site peptide was further purified by offline HPLC before subjecting it to LC-MS/MS on a quadrupole mass spectrometer using electrospray ionization. The method successfully identified the pesticide-adducts from

butyrylcholinesterase in four patients whose butyrylcholinesterase was inhibited by 60–84%, at the time of analysis. However, analysis of samples from two patients whose inhibition levels were 8 and 22% was unsuccessful.

Finally, chymotrypsin was used by Tsuge and Seto to release the active-site peptide from human butyrylcholinesterase modified by sarin, soman or VX.[76] The authors used procainamide affinity chromatography and SDS polyacrylamide gel electrophoresis to purify the butyrylcholinesterase from serum, and then used in-gel chymotryptic digestion to prepare the peptides for LC/MSMS analysis on a triple quadrupole mass spectrometer. Despite the notorious propensity of chymotrypsin to cleave at multiple sites, the authors were able isolate a single active-site peptide, GES*AGAASVSL, for adduct evaluation (the * marks active-site Ser198). The lowest limit of detection was calculated to be equivalent to 1% inhibition of butyrylcholinesterase.

Proteolysis of labelled protein – butyrylcholinesterase, carbamates

Less work has been done on the identification carbamate adducts. In all likelihood, this reflects the instability of carbamate adducts which makes the task of detecting them more difficult. The half-life for spontaneous reactivation of both N-methyl-carbamylated and N-dimethyl-carbamylated human butyrylcholinesterase (the two most common adducts from carbamate pesticides) is 2–4 h.[52,77]

Fidder *et al*. successfully used the same peptic proteolysis procedure that they developed to detect organophosphylated human butyrylcholinesterase (see the section on 'Proteolysis of labelled protein – butyrylcholinesterase, organophosphylates') to detect pure, pyridostigmine inhibited butyrylcholinesterase.[47] Pyridostigmine is an N-dimethyl-carbamate which has been used as a prophylactic agent for nerve agent exposure.

Sun and Lynn were successful in applying the tryptic procedure they used for pure, organophosphylated horse butyrylcholinesterase (see the section on 'Proteolysis of labelled protein – butyrylcholinesterase, organophosphylates') to detect pure, carbaryl inhibited horse butyrylcholinesterase.[75] Carbaryl is an N-methyl-carbamate.

In neither of these experiments was a low LOD determined or a retrospective time window established.

The more challenging task of detecting carbamate-adducted butyrylcholinesterase in human serum was undertaken by Li *et al*.[52] To minimize the effect of spontaneous reactivation, the authors developed a 15-min procainamide purification procedure that was terminated by boiling the partially purified preparation to denature it and stop reactivation. Reactivation is an enzyme mediated process that is stopped by denaturation. The labelled-peptide was isolated by HPLC and analyzed by LC-MSMS using multiple reaction monitoring (MRM) in combination with simple fragmentation via collision induced dissociation. A 1 ml blood sample from a victim of attempted murder and another 1 ml blood sample from a victim of attempted suicide were processed. Both victims had been poisoned with carbofuran (an N-methyl-carbamate). Butyrylcholinesterase activity in the serum of the murder victim was 83% inhibited at the time of analysis, while that from the suicide victim was 23% inhibited. Both samples yielded MRM and MSMS spectra consistent with an N-methyl-carbamate adduct at the active-site of butyrylcholinesterase. The Li method was applied a second time to samples from individuals poisoned by aldicarb (2-methyl-2-(methylthio) propanal O-(N-methylcarbamoyl) oxime) and Baygon (the trade name for 2-isopropoxyphenyl N-methyl carbamate, a pesticide produced by S. C. Johnson & Sons), both N-methyl-carbamates.[48] Inhibition of the butyrylcholinesterase activity in the serum of the aldicarb victim was 97%, while that in the serum of the Baygon victim was 22%. Analysis of the aldicarb sample was successful while that of the Baygon sample was not.

Proteolysis of labelled protein – butyrylcholinesterase, phosphate

Tri-o-cresyl phosphate is a component of tri-cresyl phosphate which is used as an additive in jet engine oil.[78] Exposure of airline passengers to fumes from the engines of jet airliners can cause a condition that is referred to as aerotoxic syndrome. The agent most commonly discussed as being responsible for aerotoxic syndrome is tri-o-cresyl phosphate, though this has not yet been proven. Other components of jet engine oil, mono-o-cresyl phosphate and di-o-cresyl phosphate cause delayed neuropathy in hens at lower doses than tri-o-cresyl phosphate.[2,78,79] Tri-o-cresyl phosphate itself is not toxic. It must be activated to 2-(o-cresyl)-4H-1,2,3-benzodioxaphosphoran-2-one by liver microsomes.[80,81] Activation appears to be mediated by liver microsomal cytochrome P-450. Injection of high doses of 2-(o-cresyl)-4H-1,2,3-benzodioxaphosphoran-2-one into chickens causes ataxia and demyelination of nerves in the spinal cord which are typical symptoms of organophosphate-induced delayed neuropathy.[81,82]

Reaction of butyrylcholinesterase with 2-(o-cresyl)-4H-1,2,3-benzodioxaphosphoran-2-one results in a unique, phospho-butyrylcholinesterase adduct[83,84] which is a convenient marker for in vivo exposure to tri-o-cresyl phosphate. Purification of butyrylcholinesterase from exposed serum using ion-exchange chromatography (Q-Sepharose, at pH 4) and procainamide affinity chromatography, followed by pepsin digestion and enrichment/concentration of the phospho-peptide using titanium-oxide affinity chromatography (Titansphere, TiO_2) creates a preparation from which as little as 0.05% phosphorylated butyrylcholinesterase can be detected using matrix-assisted laser desorption/ionization TOF-MS.[85] Testing of the serum from random airline passengers immediately after flying has shown that approximately 50% have phosphorylated butyrylcholinesterase, despite the fact that those passengers showed no signs of toxicity. That the phosphorylated butyrylcholinesterase was associated with the flight was shown by the observation that the phosphorylated butyrylcholinesterase was not present in the serum of passengers retested three months after the flight.[85]

Proteolysis of labelled protein – acetylcholinesterase

Jennings *et al*. used pure mouse acetylcholinesterase inhibited with dichlorvos, paraoxon, or diisopropylfluorophosphate for method development.[86] Tryptic digestion released the adducted and non-adducted active-site peptides which were then resolved with matrix-assisted laser desorption/ionization TOF-MS. The presence of both adducted and non-adducted products in a single mass spectrum allowed accurate determination of the fraction of acetylcholinesterase that was inhibited. Quantitation was sufficiently accurate to determine the kinetics of reactivation and aging. Both aged and non-aged products were identified. The lower limit of detection was equivalent to 5% inhibition of acetylcholinesterase. The method was applied to the

determination of adducted-acetylcholinesterase from the brain of a mouse treated with acute, sublethal levels of metrifonate (200 or 400 mg/kg injected intraperitoneally). A single mouse brain was sufficient for successful detection.

Spaulding *et al*. used pure mouse acetylcholinesterase inhibited by paraoxon or methyl paraoxon for method development.[87] Adducted and non-adducted active-site peptides were released from mouse acetylcholinesterase by chymotrypsin and analyzed by LC-MS/MS using quadrupole/time-of-flight tandem mass spectrometry(QqTOF-MS/MS).

Aging of organophosphylated adducts is an important consideration when looking for biomarkers of exposure. The most common form of aging involves loss of an alkyl side-chain from the phosphorus as depicted in Figure 4. However, Kropp and Richardson (using surface enhanced laser desorption/ionization TOF-MS on tryptic peptides) have found that human acetylcholinesterase inhibited by mipafox ages by loss of both isopropylamine side-chains (refer to Table 1 for the relevant structure).[88]

Proteolysis of labelled protein – serum albumin

Organophosphorylation of a tyrosine in serum albumin was first described by Sanger, who sequenced diisopropylfluorophosphate-labelled peptides from partial acid hydrolyses of rabbit, bovine and human serum albumin (using high voltage paper ionophoresis).[89] Peptides RYTK, RYTR and RYTK were identified for rabbit, bovine and human, respectively. Reaction of diisopropylfluorophosphate with tyrosine in human albumin was supported by Means and Wu, who analyzed changes in the UV absorbance of albumin following the reaction.[90] Forty-two years after Sanger's report, reaction of albumin with organophosphylates was confirmed by mass spectral analysis of an organophosphonylated, tryptic peptide from bovine serum albumin.[91] The labelled tyrosine was located at position 410 (Tyr411 in human serum albumin).[92] Subsequently, it was found that a number of proteins (transferrin, serum albumin, kinesin 3 C, alpha 2-glycoprotein 1 zinc, pro-apolipoprotein A-1, keratin, tubulin, actin, ATP synthase, adenine nucleotide translocase I, chymotrypsin and pepsin) could be labelled on tyrosine using a variety of organophosphylates (soman, sarin, chlorpyrifos-oxon, dichlorvos, FP-biotin and diisopropylfluorophosphate).[93] Reaction of organophosphylates with tyrosine is relatively non-specific. Multiple tyrosines on a given protein can be labelled, though one tyrosine is generally much more susceptible than the others, for example Tyr411 on human serum albumin.[94] The sensitive tyrosines are not part of a classical consensus sequence, but most are located within a few angstroms of a positively charged residue suggesting that through-space charge-charge interactions may facilitate the ionization of the phenolic hydroxyl thereby making it a better nucleophile for reaction with organophosphylates.[93]

Several groups have used the susceptibility of albumin to react with organophosphylates to design assays for detection of exposure. These are based on detection of labelled peptides generated through proteolysis with pronase, protease type XIV, pepsin, or Glu-C.

Black *et al*. were the first to use labelled-tyrosine as a biomarker for exposure to organophosphylates.[95] They digested sarin- or soman-labelled human plasma to single amino acids with Pronase E, and then analyzed the digests by mass spectrometry. Substantial quantities of organophosphonylated tyrosine were detected. Based on the fact that organophosphonylated albumin yielded labelled-tyrosine when treated with Pronase E, the authors proposed that albumin was the source of the labelled-tyrosine from treated plasma.

Williams *et al*. confirmed the appearance of organophosphylated tyrosine in pronase digests of human plasma treated with sarin, soman cyclosarin tabun or VX,[96] and extended the sensitivity of the method by employing multiple reaction monitoring mass spectrometry. The low limit of detection was equivalent to 10–20% inhibition of butyrylcholinesterase. Reaction with VX was much weaker than with the other agents, consistent with the weaker leaving group potential of its thiol leaving group. Sarin-tyrosine adducts could be detected in the blood of guinea pigs exposed to a 0.5 x LD_{50} dose of sarin (injected subcutaneously) for up to 24 days after injection. Soman and tabun adducts were detectable in guinea pigs for 7 days (the time at which the animals were sacrificed) after exposure to 5 x LD_{50} of soman and tabun, despite pre-dose treatment with pyridostigmine and post-dose treatment with oximes (P2S, HI-6 or toxogonin), anticholinergic agents (atropine or hyoscine) and anticonvulsants (avizafone or midazolam).

Read *et al*. further modified the method of Black by increasing the concentration of the digestion buffer to better control the pH, changing from Pronase E to Protease type XIV, and using C8 or C18 SPE to clean and concentrate the organophosphylated-tyrosine.[45] With these improvements, the low limit of detection was reduced to a level equivalent to 10% inhibition of butyrylcholinesterase. The authors examined the retrospective capability of the assay using marmosets that had been exposed to 2 x LD_{50} amounts of sarin, soman, cyclosarin or tabun followed by treatment with the oxime HI-6. Tyrosine adducts (non-aged) were detected 24 days after exposure. This was contrasted to nonapeptide adducts of butyrylcholinesterase from the same animals, assayed by the method of Fidder, which could not be detected 24 days after exposure.

Li *et al*. used pepsin to digest organophosphylated pure human albumin.[92] A pair of peptides (VRY*TKKVPQVSTPTL and LVRY*TKKVPQVSTPTL) that contained Tyr411 (marked by *) were obtained. Albumin labelled with dichlorvos, chlorpyrifos oxon, diisopropylfluorophosphate or sarin gave strong signals in the matrix-assisted laser desorption/ionization TOF mass spectrometer after dilution of the digest to 0.7 μM. The low limit of detection was 70 nM labelled albumin. Labelled peptic peptides were also obtained from organophosphylated human plasma, though it was necessary to dilute the plasma digest 1000-fold before analyzing the sample by MALDI-TOF mass spectrometry.[92] Reaction of albumin with chlorpyrifos oxon appeared to be slower than with dichlorvos, requiring approximately10-times higher concentration to obtain the same amount of labelling. This procedure was used to detect soman-labelled peptides from human serum (in vitro).[97] The method was ultimately applied to samples from people who had either been accidentally exposed to dichlorvos or who had attempted suicide by consuming dichlorvos.[48] Organophosphorylated albumin peptides could be detected in the serum from victims whose butyrylcholinesterase activity was inhibited by 80–84% at the time of analysis, but adducted-peptides could not be detected when the butyrylcholinesterase activity had been inhibited by only 8%. In all cases, the identity of the organophosphylated peptides was confirmed by multiple reaction monitoring and analysis of fragmentation spectra obtained by collision induced dissociation using electrospray-ionization, tandem-quadrupole mass spectrometry.

Tarhoni *et al*. used a competitive binding assay to assess the relative reactivity of a variety of organophosphates to albumin

(azamethiphos oxon, chlorfenvinphos oxon, chlorpyrifos oxon, diazinon oxon, pirimiphos-methyl and malaoxon).[98] Azamethiphos oxon and malaoxon reacted more poorly with albumin in rat plasma than the others, both in vitro and in vivo, making it apparent that organophosphates that possess an ester linkage to the leaving group are more reactive than those that posses a thioester.

John *et al*. developed a similar assay based on proteolysis by Glu C.[99] Human plasma albumin was reacted with an array of organophosphylate pesticides and nerve agents. The albumin was extracted using a ProteoExtract albumin removal column (Calbiochem), reduced and alkylated, digested with Glu C and analyzed by matrix assisted laser desorption/ionization TOF-MS. The presence of organophosphylate on labelled peptides was confirmed by analysis of post-source decay fragmentation spectra. V-type agents were less reactive than G-type agents.

Noort *et al*. used pepsin and pronase to characterize the binding of a series of organophosphothioates (malathion, parathion, chlorpyrifos, fenitrothion and pirimiphos-methyl) to human serum albumin.[100] After labelling, the authors extracted the albumin from plasma with a HiTrap Blue HP affinity column (GE Healthcare), digested the albumin and analyzed the peptides with a QqTOF mass spectrometer. Fragmentation of the adducted, peptic peptide via collision induced dissociation revealed labelling on Tyr411 in the peptide LVRY*TKKVPQVSTPTL (where * indicates the labelled tyrosine). Pronase yielded a labelled tyrosine amino acid. Low limits of detection depended on the organophosphothioate that was used. Parathion and chlorpyrifos-oxon could be determined at 1 μM while fenitrothion and pirimiphos-methyl were limited at 1 mM and malathion required >1 mM to detect.

Summary – pros and cons for the various assays

Each of the methods for monitoring exposure has its advantages. None of the methods fulfills all of the criteria for an ideal biomarker. The ideal biomarker should be present in an easily obtainable matrix, should be relatively long-lived, should provide unequivocal identification of the agent, and should be detectable at low concentrations. Each method uses an easily obtainable matrix, urine or blood. Only some of them can be applied at relatively long times after exposure. In many cases, only direct detection of the toxicant provides unequivocal identification of the agent. Most have a low LOD around 10 nM, although the low LOD for fluoride regeneration is 0.04 nM. Refer to Table 2 for a summary of the properties of each method.

Monitoring inhibition of butyrylcholinesterase/acetylcholinesterase activity is the simplest method, but it is semi-quantitative, does not provide any information on the identity of the toxicant and has limited use for retrospective studies. Inhibition levels are compromised by oxime therapy. However acetylcholinesterase in red cells mirrors acetylcholinesterase activity in the synapses and is a reliable means of following the course of treatment. Assays can be performed by uninitiated personnel after minimal training.

Direct detection of the intact toxicant provides definitive information on the identity of the toxicant, but assays must be performed within minutes to hours of exposure because the toxicants are rapidly hydrolyzed or metabolized and cleared from the circulation. Intact toxicants can be degraded by oximes. Sensitivity is in the mid-range. Analysis relies on GC which complicates sample preparation and requires highly trained personnel.

Monitoring the metabolites extends the time after exposure during which retrospective analysis can be successfully performed, relative to 'direct detection of the intact toxicant'. Metabolites are hydrolyzed forms of the original toxicant, therefore structural information is lost during metabolism making true identification of the original toxicant ambiguous. Like 'direct detection', sensitivity is in the mid-range. The assay is unaffected by oximes. Interpretation of a positive result can be ambiguous because even though detection of metabolites in the urine (or blood) can be an indication of exposure to an active pesticide, it can also be an indication of simple exposure to naturally occurring hydrolysis products. Both GC-MS and LC-MS methods have been adapted to this technique. Highly trained personnel are required.

Fluoride reactivation is the most sensitive method, being about 500-fold more sensitive than butyrylcholinesterase activity assays and 50-fold more sensitive than 'direct detection'. However, maximum sensitivity requires prior knowledge of the nature of the toxicant so that the proper parameters can be entered into the multiple reaction monitoring method. Aging prevents analysis. Oxime therapy compromises analysis in cases where the therapy displaces the phosphyl moiety, e.g. from cholinesterases. Organophosphylated albumin can still provide information after oxime therapy. For both albumin and butyrylcholinesterase, formation and release of the adduct results in loss of structural information which makes specific identification of the original toxicant ambiguous. The method employs gas chromatography. Retrospective potential is good when incubation is performed at 40 °C for 2 h to release the toxicant from albumin-adducts as well as butyrylcholinesterase adducts. Withdrawing blood immediately after exposure and freezing the samples makes it possible to perform retrospective analysis years after exposure.

Trypsin/alkaline phosphatase release of the adduct is not limited by aging, however structural information is lost upon formation of the adduct, thereby limiting the identification of the original toxicant. Analysis is compromised by oxime therapy. The method employs LC which simplifies sample preparation but highly trained personnel are still required.

Butyrylcholinesterase peptide analysis is capable of identifying both aged and non-aged adducts, thereby extending the time-period after exposure during which retrospective analysis can be performed, regardless of the rate at which the adducts age. Structural information is lost upon forming the adduct making identification of the original toxicant ambiguous. This is the only method that has been successfully applied for the determination of carbamate exposure in people. The technique displays mid-range sensitivity sufficient for use in forensic studies on people who have been exposed to moderate and large doses of toxicant. Analysis is limited by oxime therapy. The method employs LC which simplifies sample preparation but highly trained personnel are still required.

Acetylcholinesterase peptide analysis can also be used to identify both aged and non-aged adducts, thereby extending the scope of retrospective analysis. However the length of time after exposure during which the presence of the adduct can be detected was not examined. Again, structural information on the toxicant is lost upon adduct formation making identification of the original toxicant ambiguous. The technique displays mid-range sensitivity and analysis is limited by oxime therapy.

Table 2. Assays for detection of exposure to organophosphylates: pros and cons.

Assay	Pros	Cons	Retrospective window [a]	Low limit of detection	
				Conc. nM	Equivalent to % BChE inhibition [b]
Inhibition of AChE/BChE [c] Activity	Cheap, simple, rapid and convenient assay that can be conducted by personnel with little experience Detection equipment is portable AChE activity in red cells mirrors AChE activity in peripheral synapses Applicable to high-throughput Determination of exposure does not require prior knowledge of the toxicant	Nature of the toxicant is not identified Not reliable for inhibition less than 20% Large individual-to-individual variability Activity level is affected by pregnancy, diseases and medications Inhibition is reduced by oxime therapy Denaturation destroys activity	???	10	20
Direct Detection of Toxicant	Allows specific identification of toxicant Mass spectral fragmentation provides confident identification	Shortest retrospective analysis window Gas chromatography complicates sample preparation Analysis equipment is expensive Highly trained personnel required	minutes to hours	1	2
Detection of the Metabolites of the Toxicant	Mass spectral fragmentation provides confident identification	Short retrospective analysis window Gas chromatography complicates sample preparation Hydrolysis of toxicant results in lost identification information Hydrolyzed metabolites can occur naturally, leading to false positives Analysis equipment is expensive Highly trained personnel required	2-3 days generally up to 14 days occasionally	5	10
Fluoride Reactivation	Highest sensitivity Retrospective window is long Uses liquid chromatography which simplifies sample preparation	Adduct formation results in loss of identification information Some prior knowledge of the toxicant is required for the most sensitive analysis Reactivation is prevented by aging and compromised by oxime therapy Analysis equipment is expensive Highly trained personnel required	50 days [d]	0.02	0.04

Trypsin/ Alkaline Phosphatase of AChE [b]	Aged adducts can be detected Uses liquid chromatography which simplifies sample preparation	Adduct formation results in lost identification information Analysis is compromised by oxime therapy Analysis equipment is expensive Highly trained personnel required	100 days [e]	??	??
BChE [b] Peptides	Aged adducts can be detected Large retrospective window BChE reacts with all nerve agents and pesticides Has been used for detection of carbamate exposure	Adduct formation and aging of the adduct results in lost identification information Some prior knowledge of the toxicant is required for the most sensitive analysis Analysis is limited by oxime therapy Analysis equipment is expensive Highly trained personnel required	21-35 days	1-7	2-15
AChE [b] Peptides	Aged adducts can be detected Large retrospective window AChE reacts with all nerve agents and pesticides	Adduct formation and aging of the adduct results in lost identification information Analysis is limited by oxime therapy Analysis equipment is expensive Highly trained personnel required	100 days [e]	4	8
Serum Albumin Peptides	No aging Adducts are stable to oximes and hydrolysis Large retrospective window Adducts are formed by both phosphothioates and phosphates	Adduct formation results in loss of identification information Significant reaction occurs only for the more reactive OP Analysis equipment is expensive Highly trained personnel required	at least 24 days	5	10

[a]'Retrospective Window' refers to the length of time after exposure that the presence of toxicants can be detected.
[b]Percentage of butyrylcholinesterase activity is based on 50 nM butyrylcholinesterase in serum.[40]
[c]BChE stands for butyrylcholinesterase; AChE stands for acetylcholinesterase.
[d]Reactivation is performed at 40 °C for 1 h.
[e]The retrospection window is assumed to be the same as the life time of the red cell in circulation which is about 120 days.

Analysis employs LC which simplifies sample preparation but highly trained personnel are still required.

Serum albumin peptide-adducts are stable to hydrolysis, resistant to oxime therapy and do not age thereby providing them with large retrospective potential. Adducts can be formed by both organophosphorothioates and organophosphylates, however reaction is poor with members of either class of compound if they possess a thioester leaving group (such as VX or malathion). Sensitivity is poor, corresponding to 10–20% inhibition of butyrylcholinesterase. As is common for peptide adducts, adduct formation results in loss of structural information necessary to definitively identify the original toxicant. The method employs LC which simplifies sample preparation but highly trained personnel are still required.

Future directions

Antibodies for detection of exposure

Antibodies directed against organophosphylated tyrosine could be developed. These antibodies are expected to distinguish between exposure to soman, sarin, and pesticides because adducts on tyrosine do not age. For example, soman bound to tyrosine does not lose the pinacolyl group. Antibodies to organophosphylated serine could also be developed, but will require an antigen that is incapable of losing the organophosphate in a beta-elimination reaction or by the action of phosphatases. Such an antigen could be prepared by use of a di-fluorinated organophosphorylated serine mimic.[101] In a case where antibodies failed to be produced against phosphorylated serine because of phosphatase-mediated dephosphorylation of the antigen, use of a phosphorylated serine mimic successfully yielded high titers of specific antibodies. Organophosphate-specific antibodies would be useful not only for detection of exposure, but also for understanding the mechanism of illness from low dose exposure. It is likely that many proteins in addition to the cholinesterases and albumin are modified by organophosphorus poisons. Specific antibodies could be used to immunoprecipitate these proteins for identification by mass spectrometry.

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