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Review

History and perspectives of bioanalytical methods for chemical warfare agent detection☆,☆☆

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ABSTRACT

This paper provides a short historical overview of the development of bioanalytical methods for chemical warfare (CW) agents and their biological markers of exposure, with a more detailed overview of methods for organophosphorus nerve agents. Bioanalytical methods for unchanged CW agents are used primarily for toxicokinetic/toxicodynamic studies. An important aspect of nerve agent toxicokinetics is the different biological activity and detoxification pathways for enantiomers. CW agents have a relatively short lifetime in the human body, and are hydrolysed, metabolised, or adducted to nucleophilic sites on macromolecules such as proteins and DNA. These provide biological markers of exposure. In the past two decades, metabolites, protein adducts of nerve agents, vesicants and phosgene, and DNA adducts of sulfur and nitrogen mustards, have been identified and characterized. Sensitive analytical methods have been developed for their detection, based mainly on mass spectrometry combined with gas or liquid chromatography. Biological markers for sarin, VX and sulfur mustard have been validated in cases of accidental and deliberate human exposures. The concern for terrorist use of CW agents has stimulated the development of higher throughput analytical methods in support of homeland security.

Contents

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1. Introduction

Bioanalytical methods for chemical warfare (CW) agents and their biological markers (biomarkers) of exposure have applications in four main areas:

- Toxicokinetic studies, primarily in support of the development of medical countermeasures.
- Forensic investigations of allegations of CW use.
- Diagnosis of exposure, to ensure appropriate medical treatment.
- Monitoring of workers engaged in activities such as demilitarisation.

All of the CW agents listed in Schedule 1 of the Chemical Weapons Convention (CWC) (nerve agents and vesicants, but excluding ricin and saxitoxin) [1] are electrophilic species, with varying degrees of reactivity with water and biological nucleophiles. The structures of the main Schedule 1 agents, plus the incapacitant BZ (Schedule 2), are shown in Fig. 1. These agents have relatively short lifetimes in the human body, being hydrolysed, metabolised, or covalently bound to nucleophilic sites on macromolecules such as proteins and DNA. Metabolites and covalent adducts provide biomarkers of exposure, which find application in forensics, diagnosis and monitoring [2–6].

This paper provides a short historical overview of the development of bioanalytical methods for CW agents, plus a more detailed overview of methods developed for organophosphorus (OP) nerve agents. The emphasis is on biomarkers of exposure rather than methods used for research, e.g. in toxicokinetic studies.

2. Historical perspectives

2.1. Samples

Most of the research on bioanalytical methods for CW agents and their biomarkers has been directed at blood (whole, plasma, serum or red cells) and urine. These are the most accessible samples that accumulate a high proportion of the dose in surviving casualties. Blood has the disadvantage that collection requires the intervention of medical personnel, and it requires careful handling. Breath, saliva and hair appear to have attracted only exploratory studies, although external contamination of hair with sulfur mustard was demonstrated in a United Nations investigation of CW by Iraq against Iran [7].

2.2. Analysis of unchanged agents in blood/plasma

Initial research into bioanalytical methods focused mainly on unchanged nerve agents in blood or plasma. These methods were required for toxicokinetic/toxicodynamic studies, to provide quantitative data on which to base medical countermeasures [8,9]. They evolved from an increasing appreciation during the 1970s and 1980s of the different biological activities of enantiomers of nerve agents, both in terms of their potency as inhibitors of the enzyme acetylcholinesterase (AChE), and their scavenging and metabolic detoxification by esterases and hydrolases in the body [10]. The rapid development of commercial gas and liquid chromatography during the 1980s, particularly in combination with mass spectrometry, provided the tools for sensitive analytical methods, including those that differentiated enantiomers. Although analysis for unchanged agents could have diagnostic and forensic applications, in most cases blood would have to be sampled within a few hours of an exposure for there to be significant levels of residual free agent.

2.3. Metabolites as biomarkers of exposure

The initial impetus for the development of bioanalytical methods for verification and diagnosis arose from allegations of CW use in the 1980s, firstly in Southeast Asia, secondly in the Iraq–Iran conflict, and shortly afterwards in the internal conflict between Iraq and its Kurdish population. The alleged use of trichothecene mycotoxins in Laos and Kampuchea in the early 1980s, the so-called 'yellow rain' [11], was supported by the trace analysis of environmental and biomedical samples [12]. However, a number of inconsistencies led to a prolonged, and at times acrimonious, debate on the validity of the analyses and the conclusions drawn [13]. One particular debate concerned the levels of unmetabolised toxins found in the blood and urine of alleged casualties, in some cases more than two months after a reported attack. A study of trichothecenes such as T-2 toxin in human plasma in vitro indicated a half-life in the region of 10 h or less [14]. Toxicokinetic studies across a number of species, e.g. [15], indicated that metabolism was rapid and that the half-life of T-2 and other trichothecenes in blood in vivo was likely to be of the order of minutes.

Shortly after this, allegations of CW use arose from the Iraq–Iran conflict, initially concerning the use of sulfur mustard by Iraq [7]. Urine and blood samples were collected from Iranian casualties of vesicant poisoning being treated in hospitals in Ghent and Utrecht [16], but no validated bioanalytical methods were available. The first analyses of these samples focused on thiodiglycol, the simple hydrolysis product of sulfur mustard [17,18]. The method used hydrochloric acid to convert thiodiglycol back to sulfur mustard, which was analysed by headspace GC–MS. The analyses successfully identified elevated levels of thiodiglycol in urine from exposed individuals but also found quite variable background levels of the hydrolysis product in urine from non-exposed subjects. Low background levels of thiodiglycol and its sulfoxide have since been reported by other laboratories, e.g. [19,20].

It was apparent that if biomedical sample analysis was to be used for verification then much greater knowledge of the biological fate of CW agents was required. It was also acknowledged that laboratories engaged in this type of work needed to introduce rigorous controls into their analytical methods and adopt forensic standards. This was probably the point at which serious attention was directed towards the development of analytical methods for biomedical samples for evidence of exposure to CW agents.

There followed a metabolite study of sulfur mustard [21], which built on previous studies undertaken before the availability of analytical mass spectrometry [22,23]. More definitive metabolite biomarkers of exposure to sulfur mustard (derived from the β-lyase pathway) were identified [21] and sensitive analytical methods based on GC–MS–MS were developed for their detection [19]. These methods were applied retrospectively to stored samples from Iranian casualties, to stored samples from two Kurdish casualties [19], and to samples from two subjects accidentally exposed to sulfur mustard from a World War 1 munition [24]. β-Lysase metabolites were detected in all samples, and control samples gave true blanks.

The nerve agent tabun was also reported to have been used extensively during the Iraq–Iran conflict [7], but no bioanalytical methods existed for confirming an exposure to this nerve agent until many years later, particularly as its initial hydrolysis products are unstable (see below). Sarin was used against the Kurdish community in Iraq, substantiated by the analysis of environmental samples [25], but no biomedical samples appear to have been available from Kurdish casualties of nerve agent attacks.

A new threshold was crossed in 1994 and 1995 when the Aum Shinrikyo sect released sarin in Matsumoto City and in the Tokyo subway [26]. The primary hydrolysis product of sarin, isopropyl methylphosphonic acid, was detected in samples from both incidents. Metabolites of VX derived from hydrolysis were detected

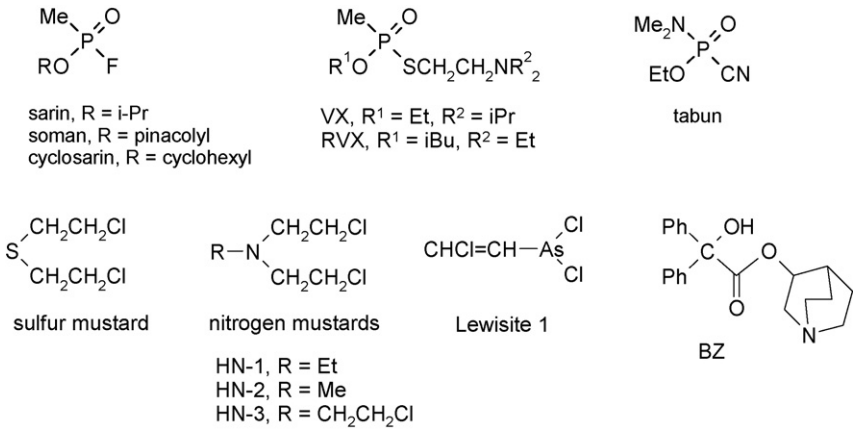

Fig. 1. Structures of the main Schedule 1 chemical warfare agents of concern (RVX = Russian V agent), plus BZ (Schedule 2).

in blood and urine from an assassination victim of the sect. These analyses are discussed in greater detail below.

Over the past two decades sensitive analytical methods based on GC–MS and LC–MS, many using tandem mass spectrometry (MS–MS), have been reported for hydrolysis products of the vesicants Lewisite and nitrogen mustards (HN-1, HN-2, HN-3), nerve agents and the incapacitant BZ in urine and blood. The trend has been towards the increasing use of LC–MS rather than GC–MS, thus avoiding the need for derivatisation, and MS–MS which generally provides lower limits of detection than single stage MS, combined with a greater selectivity. These methods have been reviewed [2,4–6]. The US Army published a technical bulletin in 1996 providing details of assays for detecting exposure to sulfur mustard, nerve agents and hydrogen cyanide [27].

2.4. Covalent adducts with macromolecules as biomarkers of exposure

During the 1980s there was increasing concern for exposure of workers and other vulnerable groups to environmental or industrial carcinogens. Many of these chemicals are simple electrophiles, e.g. methyl bromide, ethylene oxide, and therefore have some chemical similarities to CW agents. Metabolites provide evidence only of recent exposure and an increasing amount of research was being directed at identifying longer lived and cumulative biomarkers that could be used for monitoring chronic or sub-chronic exposure. Examples were alkylated haemoglobin, alkylated albumin and alkylated DNA [28,29]. This work, together with the lack of methods available for analyzing samples from Iranian casualties, stimulated a similar search for longer lived biomarkers of CW agents. Efforts at TNO, Rijswik, The Netherlands and the Defence Science and Technology Laboratory (Dstl), Porton Down, UK were directed initially at sulfur mustard. Adducts were identified in vitro, with nucleophilic amino acid residues on haemoglobin (N-terminal valine, histidine, aspartic and glutamic acids) [30,31], albumin (cysteine) [32], and with DNA [33]. Following the development of analytical methods, based on GC–MS, GC–MS–MS, LC–MS–MS, and immunoassay (DNA), all of these adducts have been detected retrospectively in stored blood samples from Iranian casualties [34,35], and/or in samples from accidental human exposures [35,36].

In the 1990s TNO focused on nerve agents and developed versatile methods that identify phosphylated butyrylcholinesterase (BuChE), or AChE, after displacement of the OP moiety with fluoride ion [37], or as adducted peptides after enzymatic digestion [38]. These methods were successfully applied to samples collected from casualties of the Matsumoto and Tokyo terrorist releases of sarin [37,38]. All of the methods described above are targeted at a limited number of specific analytes, unless full scan or time of flight mass spectrometry can be used. TNO have recently developed two generic methods for identifying BuChE adducts, one that detects all CWC Schedule 1 nerve agents [39] and one that detects BuChE inhibited by any OP [40]. In 1999 Dstl reported tyrosine adducts on albumin as potential alternative biomarkers of nerve agents [41] and later demonstrated their occurrence in guinea pigs and marmosets in vivo [42,43]. These nerve agent adducts are discussed in greater detail below. Protein adducts have also been identified for HN-2, and phosgene, as reviewed in [3–6].

2.5. The concern for terrorist use of CW agents

Until the mid-1990s, research on biomedical sample analysis of CW agents was restricted mainly, but not exclusively, to laboratories in the Netherlands (TNO), UK (Dstl) and US (USAMRICD). The release of sarin in Matsumoto City in 1994, and in the Tokyo subway in 1995, by the Aum Shinrikyo sect, and later the events of 11 September 2001, increased concern for terrorist use of CW agents. A number of additional laboratories became involved in biomedical sample analysis for CW agents, mostly in support of homeland security, which further stimulated the development of analytical methods, particularly for nerve agents. Several of these laboratories had requirements for a greater throughput than is normally required for allegations of CW use, and a number of methods were modified, simplified and/or automated [44,45].

2.6. The Organisation for the Prohibition of Chemical Weapons (OPCW)

The OPCW has an extensive network of laboratories designated for the analysis of environmental and man made samples for scheduled CW agents, their precursors and degradation products. Rigid criteria have been developed for identification and reporting, and laboratory proficiency is tested at least annually by the analysis of samples such as soil, water and organic liquids, spiked at concentrations usually in the range 5–50 ppm. At this level full scan mass spectrometry is employed; trace analytical techniques such as selected ion or multiple reaction monitoring are not required. In cases of allegations of CW use, the CWC provides for the collection of both environmental and biomedical samples [46], but observers have noted that the OPCW has no system of laboratories designated for biomedical sample analysis. In 2004, the Scientific

Advisory Board (SAB) to the Director General of the OPCW convened a Temporary Working Group, with the objective of assessing the feasibility of organising a separate system of laboratories for the analysis of biomedical samples. Recommendations for a gradual progression towards a designated laboratory system were made to the Director General in 2007. It was recognized that the first part of the process should be to broaden the number of laboratories with the knowledge and expertise to undertake such analysis through a series of confidence building exercises. An important component of these exercises would be to develop criteria for an unequivocal identification in terms of the specificity of the biomarker and the trace analytical method. These criteria would need to be broadly consistent with international practice in other areas where legislation is supported by trace analysis, e.g. in sports doping and food contamination. The first confidence building exercise is planned for November 2009.

3. Bioanalytical methods for nerve agents in blood and plasma

Bioanalytical methods for unchanged nerve agents have been applied primarily in toxicokinetic and toxicodynamic studies. Toxicodynamics provide the basis for a rational approach to medical countermeasures, and toxicokinetic parameters have implications for the timing and/or formulation of medical countermeasures [7,8].

Factors that influence the lifetime of nerve agents in vivo include chemical stability, susceptibility to hydrolytic enzymes, covalent binding to macromolecules, redistribution into tissues, and rates of absorption, metabolism and excretion. The extent to which each occurs varies with agent, species and route of exposure. Nerve agents undergo slow spontaneous hydrolysis in the aqueous physiological environment, and more rapid enzymatic hydrolysis by endogenous phosphoric triester hydrolases such as paraoxonase [47]. They are further detoxified through scavenging by irreversible binding to serine esterases such as BuChE (serum cholinesterase) and carboxylesterases [48], and binding to other proteins and macromolecules such as albumin.

Serious interest in the toxicokinetics of nerve agents commenced in the 1980s following the observation that soman, analysed as a racemate, was more persistent in experimental animals than other nerve agents such as sarin [49]. At the same time there was a growing understanding of the importance of enantiomers in nerve agent toxicology, and the role of esterases in their detoxification. Pairs of isomers, enantiomeric about the phosphorus atom, showed that high inhibitory activity vs AChE, and toxicity, resided mainly in one isomer, although this was less pronounced with VX [10]. In contrast, the less active enantiomers appeared to be more susceptible to hydrolysis by phosphoric triester hydrolases such as paraoxonase [50–52].

Route of exposure also has a major effect on the persistency of agents in the body. The main hazard from volatile nerve agents such as sarin is inhalation of vapour, where absorption through the lungs occurs in minutes. Conversely, the main hazard from low volatility V agents is likely to be from cutaneous exposure, where absorption may occur over several hours.

The analytical requirements for toxicokinetic studies are:

- Low limits of detection (LODs)—down to toxicologically relevant levels.
- Resolution of enantiomers.
- High selectivity (though this is less important than in forensic applications).
- Accurate quantitation.
- High sample throughput.

The development of analytical methods was steadily advanced during the 1980s and 1990s by the evolution of suitable analytical tools, for example, robust capillary GC columns combined with sensitive element specific detectors, e.g. nitrogen-phosphorus (NPD), flame photometric (FPD) and MS, commercially available chiral GC and LC columns, two dimensional GC, large volume injection techniques, and more recently sensitive LC–MS–MS instrumentation. Chirasil-L-Val and β or γ-cyclodextrin columns have been mostly used for chiral GC columns, and Chiralcel OD for LC. Examples of methods that have been used or proposed for toxicokinetic studies include:

- 2D GC with large volume sample introduction (thermal desorption, cold trap) NPD or GC–MS (sarin, soman) [8,9].
- Chiral GC (γ-cyclodextrin)–MS–MS (cyclosarin) [53].
- Chiral LC (Chiralcel OD)–electrochemical detection with off-line GC-FPD (VX) [54].
- Chiral LC (Chiralcel ODH)–MS–MS (atmospheric pressure CI) (VX) [55].
- Chiral GC (β-cyclodextrin)–MS (NH_3 CI) (tabun) [56].

An additional problem with analyzing nerve agents in blood or plasma is preservation of the nerve agent from further enzymatic breakdown, scavenging or regeneration. Enzymatic hydrolysis can be suppressed by immediate addition of acetate buffer at pH 4. Saturable binding sites such as AChE, BuChE and carboxylesterases can be blocked by addition of a second ChE inhibitor such as neopentyl sarin. Aluminium sulfate is added to complex fluoride ions, which can regenerate nerve agent from binding sites [8].

4. Metabolites as biomarkers of nerve agent exposure

The analytical requirements for biological markers of exposure are different from those for toxicokinetics. The main requirements are:

- Low LODs.
- High specificity is paramount if the results are to be used for forensic purposes.
- Two analytical methods or two biomarkers per agent are desirable, one for confirmation.

The lower the LODs, the longer a metabolite is likely to be detectable after an exposure, particularly as the later phase of excretion may be prolonged but at very low, possibly sub-ppb, concentrations. Some of this may be from breakdown of protein adducts, for example carboxylesterases tend to slowly reactivate. The advantages of sub-ppb LODs was demonstrated by the detection of β-lyase metabolites of sulfur mustard, using GC–MS–MS rather than single stage GC–MS, in two Kurdish casualties whose urine was collected 13 days after exposure [19].

Separation of enantiomers is not required and accurate quantitation is not usually important. High throughput is unlikely to be required in cases of alleged CW use, where the number of samples collected is likely to be small and unequivocal identification to internationally acceptable standards is the overriding requirement. High throughput may be more important in a terrorist incident, where casualty and sample numbers could be large, and particularly where methods have been adapted for diagnostic screening rather than forensics.

No detailed metabolism studies of nerve agents in vivo appear to have been undertaken, possibly because metabolism is dominated by hydrolysis and identification of minor metabolites would require additional labeling plus sensitive methods for identification.

4.1. Hydrolysis products

Nerve agents slowly hydrolyse spontaneously in the body; more rapid hydrolysis is mediated primarily by phosphoric triester hydrolases. In the case of sarin, soman, cyclosarin, VX and Russian VX, the primary hydrolysis products are alkyl methylphosphonic acids (Fig. 2). These are stable metabolites and undergo only slow further hydrolysis to methylphosphonic acid (this was however detected at moderate concentrations in casualties of the Matsumoto/Tokyo incidents [57]). Furthermore, because P–C bonds are found only in a very small number of pesticides (and not as P-methyl substituents), and in fire retardents, alkyl methylphosphonic acids are generally regarded as unequivocal biomarkers of nerve agent exposure. No background levels have yet been reported in non-exposed subjects [58].

Shih et al. [59] determined the excretion profiles of alkyl methylphosphonic acids from sarin, soman and cyclosarin in the rat following subcutaneous administration (dose 0.075 mg/kg). Urinary excretion over the first 24 h accounted for approximately 90% of the administered doses of sarin and cyclosarin. Soman was eliminated more slowly with a biphasic elimination curve; approximately 50% was excreted within the first 24 h, rising to 62% after 7 days. The first phase of elimination is due to rapid enzymatic hydrolysis of the inactive P(+) isomers by phosphoric triester hydrolases; the second phase is from slower hydrolysis of the active P(−) isomers [9].

Tabun presents a problem because its initial hydrolysis products, ethyl N,N-dimethylphosphoramidic acid and ethyl phosphorocyanidic acid, are unstable and hydrolyse further to ethyl phosphoric acid and then slowly to phosphate. Unfortunately there is a high and quite variable background of ethyl phosphoric acid in the general population, presumably from pesticides and plasticizers [60].

4.1.1. GC–MS(–MS) analysis

Alkyl methylphosphonic acids are primary indicators of nerve agents in the environment and many analytical methods have been reported. These were initially based on GC–MS and GC–MS–MS after derivatisation, but in the last decade equally sensitive LC–MS–MS methods have been reported. Single stage GC–MS can achieve LODs in the low ppb range, but for sub-ppb LODs MS–MS is required. MS–MS also provides a higher degree of specificity.

The derivatisation of nerve agents for GC–MS analysis was reviewed by Black and Muir [61]. Trimethylsilyl, *tert*-butyldimethylsilyl and methyl esters are most commonly used for environmental analysis of phosphonic acids, and can provide LODs in biomedical samples in the low ppb range. GC–MS methods based on silylation were adapted for the analysis of biomedical samples associated with the Matsumoto and Tokyo terrorist incidents, e.g. [62,63]. Lowest detection limits are achieved with pentafluorobenzyl esters, in combination with negative ion chemical ionisation (NICI) GC–MS–MS, e.g. [64–67]. Disadvantages of pentafluorobenzyl derivatives are that sample preparation is rather tedious, some procedures using solid phase extraction (SPE) for clean up both before and after derivatisation [65,67]. Maintaining system blanks down to the very low LODs can be a problem. Analytical methods also differ in the isolation of the phosphonic acid prior to derivatisation. Anion exchange [65], C_2/C_{18} reversed phase SPE [64] and polymeric SPE [67] after acidification, and normal phase non-bonded silica (for LC–MS) [58] have been reported to give good recoveries. Non-bonded silica gave less interference in LC–MS–MS analysis compared to anion exchange SPE at pH 1 [58]. Miki et al. [66] used Ag^+-form cation exchange to remove chloride ions from urine prior to extractive derivatisation under phase transfer conditions. LODs in the range 0.1–0.5 ppb were reported for pentafluorobenzyl esters in combination with polymeric SPE and NICI [67]. This method was able to detect pinacolyl methylphosphonic acid in a rhesus monkey eleven days after intoxication with 0.5× median intramuscular lethal dose of soman. Molecularly imprinted polymers have also been used for selective extraction after partitioning of the acids from serum into acetonitrile [68]. A GC–MS–MS method, designed for higher throughput, used methyl esters and simply concentrated urine to dryness [69].

Which method should be used is dependent on circumstances. In the case of a terrorist incident, biomedical samples will probably be available within hours of the exposure and metabolite levels are likely to be in the low to mid ppb range. This was illustrated in the Matsumoto and Tokyo incidents where simple derivatisation to silyl esters provided adequate LODs for urine and blood. In an alleged use of CW in a remote conflict, certified samples are unlikely to be collected until several days after the incident, and then pentafluorobenzyl derivatives or one of the sensitive LC–MS–MS methods would be the more suitable techniques.

4.1.2. LC–MS–MS analysis

Until the last decade LC–MS had not achieved the detection limits desirable for general application to biomedical samples for allegations of CW use [70]. Noort et al. [71] demonstrated the successful use of LC–MS–MS in serum samples from casualties of the Matsumoto and Tokyo incidents, where levels of isopropyl methylphosphonic acid were in the range 2–135 ng/ml. The Centers for Disease Control (CDC), Atlanta [69,72] has recently

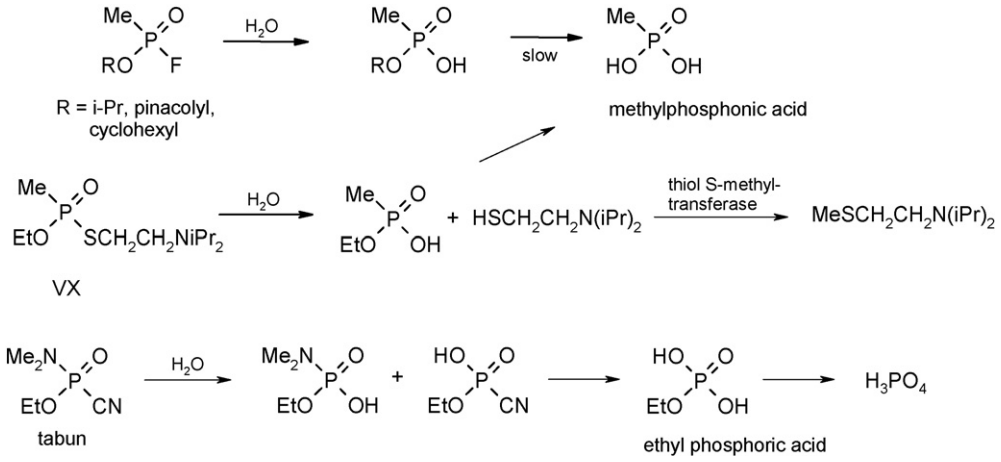

Fig. 2. Hydrolysis pathways, spontaneous and metabolic, for nerve agents.

reported highly sensitive high throughput LC–MS–MS methods, which use hydrophilic interaction liquid chromatography rather than reversed phase. In contrast to reversed phase, the HILIC column shows greater retention for the less hydrophobic acids such as ethyl methylphosphonic acid (from VX). LODs were sub-ppb, and down to 0.03 ng/ml for pinacolyl methylphosphonic acid. A high throughput method with sub-ppb LODs was also reported using reversed phase LC–MS–MS [73].

4.2. Other nerve agent metabolites

No additional metabolites derived from metabolic modification of the side chains of sarin, soman and cyclosarin have been reported; any such formation has been presumed to be negligible. The most significant knowledge gap is the metabolic fate of the dialkylaminoethylthio substituent in V agents, which is displaced on reaction with AChE and BuChE, and by hydrolysis. A metabolite $MeSCH_2CH_2N(iPr)_2$, derived from enzymatic S-methylation of this hydrolysis product, was identified in human plasma following an assassination with VX [62]. Rapid formation of this metabolite from $HSCH_2CH_2N(iPr)_2$ was confirmed in rats [74]. This metabolite has not been reported in urine; by analogy with β-lyase metabolites of sulfur mustard, it might be excreted as a sulfoxide. A significant amount of the N,N-diisopropylaminoethylthio leaving group appears to be bound to proteins such as albumin [75]. Another possibility for V agent metabolism is oxidative dealkylation of one of the N,N-dialkyl substituents, although this does not appear to have been observed. Slow O-dealkylation was reported in plasma [75].

5. Cholinesterase adducts as biomarkers of nerve agent exposure

Nerve agents inhibit the enzymes AChE and BuChE. The inhibition of AChE mediates the characteristic toxic effects of nerve agents, which result from excessive stimulation of cholinergic neurons and skeletal neuromuscular junctions. BuChE acts as a stoichiometric scavenger of nerve agents; its inhibition appears to have no significant physiological effects in the absence of other toxicants. The inhibition results from a covalent reaction of the nerve agents with a serine -CH_2OH residue in the active sites. In the absence of reactivators such as oximes or fluoride ion, spontaneous reactivation is generally slow or negligible. The inhibited enzymes therefore provide specific and relatively long-lived biomarkers of exposure, up to the turnover of the enzyme in favorable cases.

Readily accessible AChE occurs in red blood cells, and BuChE in the plasma. BuChE is usually preferred as a biomarker because of its higher abundance (~50 nM). Screening of individuals for depressed levels of red cell AChE or plasma BuChE is regularly undertaken for scientists and other workers who handle nerve agents. Suspected casualties of nerve agent poisoning can also be screened in this way but this has limitations because of the lack of baseline values and intra and individual variability in enzyme levels ([76] and references therein).

5.1. Adducts with BuChE and AChE as specific biomarkers

TNO developed two versatile methods for the identification of agent specific phosphylated BuChE. The first is based on displacement of the OP moiety from the enzyme as a fluoridate with potassium fluoride (Fig. 3) [77].

The fluoride displacement method has the advantage of being experimentally much simpler than analyzing adducts, and is easily adaptable by laboratories lacking LC–MS–MS instrumentation. The liberated phosphono/phosphorofluoridate (the original nerve agent in the case of sarin, and cyclosarin), is isolated by solid or liquid phase extraction and detected by GC-NPD, GC–MS–(MS) or

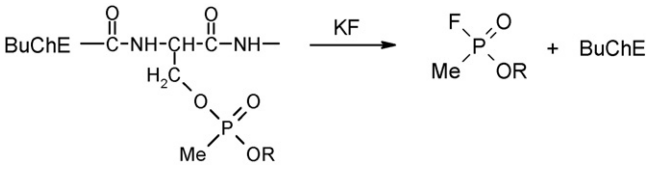

Fig. 3. Fluoride reactivation method for detecting nerve agent-inhibited butyrylcholinesterase BuChE [37].

LC–MS(–MS). The method provides a very sensitive method for detecting BuChE inhibited by sarin, cyclosarin (GF), VX and tabun; a number of modified procedures and applications have been reported, e.g. ([78] and other papers in [44,45]), and the method has been extended to red cell AChE [79]. Fluoride reactivation is much less sensitive for soman, because BuChE and AChE adducts age with loss of the pinacolyl group within minutes. The fact that the method still works with soman suggests that fluoride may be displacing the $MeP(O)OCH(Me)CMe_3$ moiety from other adducts, probably tyrosine adducts on albumin or from carboxylesterases [80]. The method was applied successfully to casualties of the Matsumoto and Tokyo sarin attacks [37], and to an accidental exposure of a laboratory worker to VX [81]. An experimentally more demanding procedure applied to Japanese casualties liberated sarin residues from red blood cell AChE as isopropyl methylphosphonic acid, after sequential digestion with trypsin and alkaline phosphatase [82].

The second method developed by TNO identifies a phosphylated nonapeptide after digestion of BuChE with the enzyme pepsin [38]:

FGES ∗ AGAAS (S∗ = phosphylated serine)

Pepsin has the advantage over trypsin of a simpler digestion procedure and the production of a shorter phosphylated peptide. BuChE is isolated from plasma using a procainamide affinity column, digested with pepsin, and the nonapeptide adduct analysed by LC–MS–MS using electrospray ionisation (ESI) and multiple reaction monitoring. The method can be used for BuChE inhibited by OP pesticides and nerve agents. Disadvantages are that it detects only aged (dealkylated) adduct with soman, and is quite laborious and technically demanding. In order to shorten the procedure TNO have developed an automated on-line digestion LC–MS–MS configuration [83].

A modification of this procedure incorporated 34 transitions into the multiple reaction monitoring programme, which allows screening for all the nerve agents covered by the generic formulae in Schedule 1 of the Annex on Chemicals in the CWC [39].

Alternative approaches to detecting phosphylated BuChE have been 1D-gel electrophoresis of isolated BuChE with in-gel chymotryptic digestion to an undecapeptide prior to LC–MS–MS [84], and MALDI-TOF MS of a 22 amino acid peptide after digestion with trypsin [85].

5.2. Adducted BuChE as a generic marker for OP exposure

The analytical methods described above are targeted at specific nerve agents. If there is no indication what, if any, nerve agent has been used, a generic screening approach could be more appropriate. TNO further developed the methodology described in 5.1 by treating the phosphylated nonapeptide with barium hydroxide, which eliminates the OP moiety from the serine residue (Fig. 4). This generates a reactive –C=CH_2 residue, which is subjected to a Michael addition with 2(3-aminopropylamino)ethanol to produce a common -$NH(CH_2)_3NH(CH_2)_2OH$ tagged serine residue irrespective of the nerve agent. The tagged nonapeptide is detected by LC–MS–MS [40].

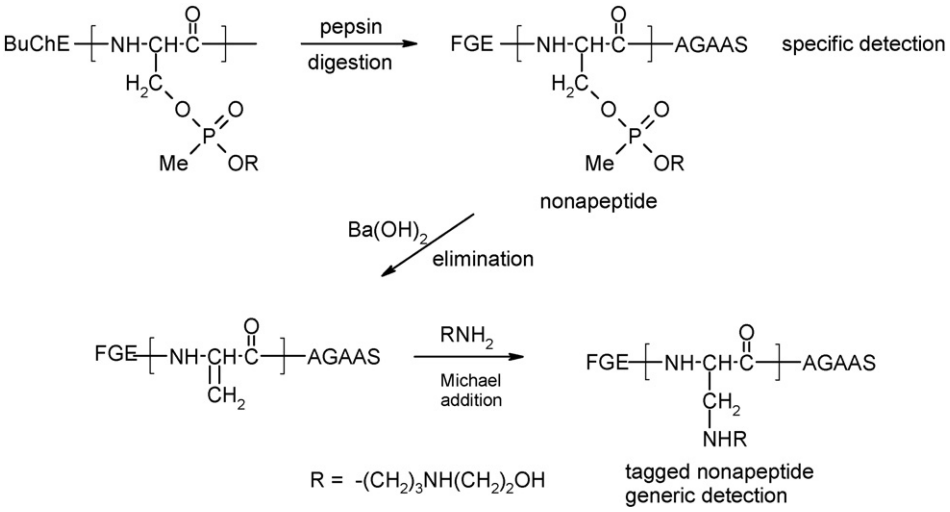

Fig. 4. Generic method for detecting organophosphorus compound inhibited BuChE [40].

6. Albumin adducts as biomarkers of nerve agent exposure

The nerve agents sarin and soman were shown at Dstl to form non-aged adducts with a tyrosine residue when incubated with human plasma, tentatively identified as the 411 tyrosine residue on albumin after isolation of a phosphonylated tripeptide (YT*K where T* is phosphonylated tyrosine) [41–43]. The site of phosphylation was confirmed as tyrosine 411 by Lockridge and co-workers using matrix-assisted laser desorption time-of-flight MS and LC quadrupole time-of flight MS–MS [86–88]. The adducts were shown not to age significantly in vitro. Tyrosine adducts were also identified for a number of organophosphorus pesticides [86,88]. Sensitive analytical methods (LODs $\leq$ 1 ng/ml) were developed for the adducts based on digestion of plasma with Pronase or Protease Type XIV, isolation of the tyrosine adducts by SPE (C_{18} or C_8), and LC–MS–MS. Non-aged tyrosine adducts were subsequently demonstrated in guinea pigs and marmosets intoxicated with sarin, soman, cyclosarin and tabun [42,43]. VX, which is chemically less reactive than phosphonofluoridates and tabun, formed an adduct in vitro in human plasma only at high concentrations [41]. Tyrosine adducts are less sensitive than BuChE as biomarkers with respect to exposure levels, but are more robust with regard to aging, particularly for soman, and displacement of the OP residue by therapeutic oximes. In blood samples obtained from medical countermeasures studies in marmosets, in which the animals were exposed to sarin, soman, cyclosarin or tabun and treated with therapeutic oximes, only tyrosine adducts were detected after 23/24 days [43]. It has been suggested that because tyrosine 411 on albumin is on the periphery of the protein, it may be a more suitable target for an immunoassay rather than inhibited ChE, where the adducted residue is located within a gorge [89]. Other, less reactive, tyrosine residues on albumin may also be phosphylated [89].

7. Future directions

Further automation and simplification of methods for metabolites can be expected. The advancement of proteomic techniques, MS instrumentation and data processing is facilitating the identification of protein adducts in complex mixtures. It may be expected that adducts of CW agents with proteins other than haemoglobin, albumin and cholinesterases will be identified. A recent paper by Tuin et al. [90] has identified additional proteins phosphonylated by a biotinylated methylphosphonofluoridate after incubation with rhesus monkey liver and cultured human A549 lung cells. Exploratory metabolomic and metabonomic studies using nuclear magnetic resonance (NMR) spectrometry have been undertaken with CW agents [91]. However these focus on the very complex biochemical status of the tissue or organism rather than on specific individual biomarkers; application to verification analysis is doubtful. NMR still does not have the required sensitivity to be used as a routine tool for detecting specific biomarkers of exposure in a CW context.

Much effort is being directed at the development of rapid and/or miniaturised kits for point-of-care diagnostics, particularly for nerve agents. A review of this work is beyond the scope of this paper but advances have been reported in a number of directions. Examples are modified assays for AChE activity, immunoassays directed towards specific adducts, more generic immunoassays for phosphylated cholinesterase, exploitation of nanotechnology, e.g. a nano-particle based electrochemical immunosensor for detection of phosphorylated acetylcholinesterase [92], and lab-on-a-chip technology, e.g. an assay that exploits fluoride reactivation of inhibited ChE and measures the AChE inhibitory activity of the fluoridate so produced [93].

8. Conclusions

Metabolite biomarkers are available for most of the previously weaponised Schedule 1 CW agents although some gaps remain. Knowledge of the metabolism of nitrogen mustards is lacking, and of particular note to this issue is the incomplete knowledge of the fate of the dialkylaminoethylthio substituent in V agents. With the single exception of a metabolite identified in the blood of an assassination victim, all of the other biomarkers for V agents identify only the alkyl methylphosphonyl part of the molecule. It can be argued that the same is true for phosphonofluoridates but it would be reasonable to assume that the leaving group is fluoride in most cases.

Protein and/or DNA adducts have been identified for most of the previously weaponised Schedule 1 CW agents, at least four protein adducts and the DNA adduct in the case of sulfur mustard. BuChE or AChE provide versatile biomarkers for nerve agents, complemented by tyrosine adducts on albumin. Each has limitations. The advantage of BuChE adducts is that they are formed with all nerve agents and can provide a very sensitive biomarker, particularly if the fluoride reactivation assay is used. A disadvantage is that rapid aging of some BuChE adducts results in the loss of structural information on the nerve agent. BuChE adducts that age only slowly may

be substantially reversed if oxime therapy has been administered, although the extent to which this may be an issue requires further investigation. A major limitation of albumin adducts is that adduct formation appears to be significant only with the more reactive nerve agents, e.g. sarin, soman, cyclosarin and tabun, and they are less sensitive biomarkers than BuChE at low exposure levels. They do however have the advantage that rapid aging does not occur, and initial studies suggest that they survive oxime therapy better than BuChE adducts. They therefore provide complementary markers, particularly for soman and tabun where rapid or partial aging of BuChE/AChE adducts occurs.

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