Analysis of Nerve Agent Metabolites from Nail Clippings by Liquid Chromatography
Tandem Mass Spectrometry

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Abstract

While several methods for the bioanalysis of nerve agents or their metabolites have been developed for the verification of nerve agent exposure, these methods are generally limited in the amount of time after an exposure that markers of exposure can be detected (due to rapid metabolism from biological matrices). In this study, a method for the analysis of nerve agent hydrolysis products from nail clippings was developed to allow evaluation of nails as a long-term repository of these markers. Pinacolylmethylphosphonic acid (PMPA) and isopropylmethylphosphonic acid (IMPA) were extracted from nail samples with N,N-dimethylformamide and subsequently analyzed by liquid chromatography-tandem mass spectrometry. Limits of detection for PMPA and IMPA were 0.3 $\mu\text{g/kg}$ and 7.5 $\mu\text{g/kg}$ and linear ranges were 0.75-300 $\mu\text{g/kg}$ and 30-1500 $\mu\text{g/kg}$, respectively. Precision was within 10% and 8% for PMPA and IMPA, respectively, and accuracy was $\pm 12\%$ for both analytes. The approach presented here is complementary to current methods for nerve agent exposure verification, and should allow for long-term determination of nerve agent poisoning.

Keywords: soman, pinacolylmethylphosphonic acid, sarin, isopropylmethylphosphonic acid, liquid-chromatography mass-spectrometry, long-term chemical warfare agent biomarker, nerve agent exposure

1. Introduction

Long-term, retrospective analysis of nerve agents is important for several scenarios. First, a low-dose exposure may cause symptoms that are easily misdiagnosed as something other than nerve agent exposure, such as pupil

constriction, rhinorrhea, mild breathing difficulties. If it is later suspected that nerve agent exposure caused these symptoms, perhaps months or even years following the exposure, the opportunity to definitively verify nerve agent exposure may be lost. This is because most agents and metabolites are eliminated from the body days after expected exposure. Another scenario necessitating long-term, retrospective detection of nerve agent exposure is the occurrence of a potential exposure in a remote area. Soldiers, contractors, and civilians in remote areas would not necessarily have access to medical treatment for weeks or months if an exposure were to occur. Moreover, medical personnel nearest the event may be ill-equipped to diagnose the cause of the symptoms, especially if patients have difficulty describing the details of the incident.

Both of these scenarios were exemplified by the unknowing demolition of chemical weapons in an open pit at Khamisiyah, Iraq during the first Iraq war [1]. It has been estimated that more than 120,000 soldiers may have encountered a vapor cloud created by the demolition [1]. Soldiers were in a remote area and those who reported to medical personnel were potentially misdiagnosed. Therefore, once nerve agent exposure was suspected, biomarkers of nerve agent exposure were lost to normal metabolism and elimination.

Several approaches have been explored to verify nerve agent exposure, including measuring the decrease in acetylcholine esterase (AChE) activity in blood [2-4], analysis of hydrolysis products of the parent agents [5-9], fluoride reactivation of bound agent [10-13], and analysis of nerve agent adducts [14-21]. A decrease in AChE activity occurs when the nerve agent binds with the active site of the enzyme

(Figure 1, Reaction A; AChE denoted by R'-OH). AChE activity is generally measured spectrophotometrically using the Ellman procedure [2, 22]. Measuring AChE activity as an indicator of nerve agent exposure has several disadvantages such as the inability to identify the parent agent, its lack of specificity for nerve agents (i.e., chemicals other than nerve agents can cause AChE inhibition), and person-to-person variability of baseline AChE concentrations. Another drawback is that *de novo* synthesis of the enzyme makes the procedure less suitable for retrospective detection of exposure [18, 22].

Nerve agents can be released from the AChE via hydrolysis (Figure 1, Reaction D), or directly hydrolyzed (Figure 1, Reaction E), to form the corresponding alkylmethylphosphonic acid (AMPA; Compound 4). AMPAs have been analyzed from several biological matrices using mainly gas chromatography (GC) or liquid chromatography (LC) with MS or tandem-MS detection. As shown in Figure 2, metabolites formed by hydrolysis are detectable for a maximum of 1 and 2 days from blood and urine [8, 23], respectively, while AMPAs in hair are detectable for at least 30 days [24].

Fluoride reactivation of enzyme bound agent (Figure 1, Reaction B) uses excess fluoride ions to cleave the phosphorus-enzyme bond, and reform the phosphorus-fluorine bond [10, 12, 25], to produce the parent nerve agent (Compound 1). Although this process is simplistic, it is highly dependent on how quickly the adduct (Compound 2) undergoes ageing (Reaction C to compound 3), after which, the adduct can no longer be reactivated [13, 15]. This process works well for analysis of sarin-esterase adducts due to relatively slow ageing (hours) [12,

13]. However, reactivation does not work particularly well for other agent adducts, such as the soman-esterase adduct, which ages more rapidly (minutes) [4]. In either case, ageing of the bound nerve agent precludes the use of this technique for long-term verification of exposure.

Nerve agent adducts, such as phosphorylated AChE or butyrylcholine esterase (BuChE) (Compound 2), are mainly analyzed by mass spectrometric (MS) methods [14, 15, 18]. If the agent adduct is hydrolyzed (Figure 1, Reaction D), long-term verification of nerve agent exposure is not possible. Although hydrolyzed adducts cannot be used to verify exposure, MS methods are still capable of analyzing aged AChE/BuChE adducts [18, 22]. Although it is currently unclear, aged adducts may offer a means of long-term verification while the adducted protein is circulating (i.e., in mammals, the half-life of AChE ranges from 40 hours to 2.84 days [26-28] and the half-life of BuChE ranges from 21 hours to 5 days [29-32]). Considering the low adduct concentrations likely to be available and the adduct half-lives, aged adducts may allow exposure verification for weeks to a few months. Other disadvantages of MS methods include requiring enzymatic digestion, arduous sample preparation, and they can be time intensive [18].

While several methods are capable of determining exposure to nerve agents, a significant problem lies in the fact that the parent agent(s) of interest, and any metabolites, are present in the body for a limited amount of time, thus decreasing the chance of detecting a past exposure (Figure 2). This problem can be resolved by identifying a new, extremely stable biomarker of exposure, or utilizing a matrix that can preserve well-known metabolites for long periods of time, allowing ample

opportunity for samples to be collected following an alleged exposure (i.e., weeks or possibly months).

Hair has shown the ability to protect markers of drugs of abuse, pesticides and pollutant exposure, and even nerve agent exposure for long periods of time [24, 33-47]. The preservation of chemical compounds in hair arises from the process of hair shaft formation. While hair cells are forming in the follicle, chemicals within the bloodstream are transferred to the hair cells. As new cells are produced in the follicle, the older cells are pushed towards the surface of the scalp, and are keratinized (i.e. the hair cells gradually die and decompose creating a dense mass while keratins (sulfur-rich proteins) create long fibers through disulfide bonds and crosslinking). During keratinization, the metabolites are trapped at the center of the hair shaft, where they are protected from further metabolism [33, 48, 49].

Long-term protection of organophosphate metabolites was demonstrated by Tutudaki and coworkers who used hair to determine chronic exposure to diazinon (an organophosphate pesticide sometimes used as a surrogate for nerve agents) in both rats and rabbits [46, 47]. Appel et al. [24] showed that a metabolite of the nerve agent soman can be detected in the hair of rats 30 days following exposure, with storage up to 5.5 years. While hair provides a matrix for extremely long-term detection of nerve agent exposure, it also has some disadvantages. One disadvantage is that hair can be regularly treated with harsh chemicals (e.g., dyes, organic solvents, or bleach). After chemical treatment, the chemical composition of the hair may change, and the analyte(s) of interest may also be compromised. Hair also has a relatively high growth rate, which increases the likelihood of removing

(i.e., cutting) and discarding the hair containing the marker of interest. Hair removal, by cutting or shaving, is also easy, and is performed quite frequently by some individuals, decreasing the chances of detecting a chemical marker.

Finger and toe nail clippings offer a complementary alternative long-term repository of nerve agent markers. To date, nail clippings have been a popular matrix to determine exposure to heavy metals [50, 51]. In a study performed by Muralidharan [50], several heavy metals, including Cd, Pb, Cr, Mn, Fe, Ni, Cu, and Zn were studied in the nails of multiple male subjects living in Mumbai for 10 years or more. This investigation used nail samples for forensic studies, and revealed several relationships between environmental exposure and health. Parizanganeh et al. [51] conducted a study in Zanjan province, Iran, where mining of lead and zinc is common. Nail samples were collected from 29 individuals in Dizajabaad village in Zanjan province. Their results showed heavy metal concentrations in the nails of subjects in Dizajabaad village were 5-50 times higher than in samples taken from individuals outside the affected area. Finger and toe nails have also been used to determine small organic molecules, to include markers of drugs of abuse [52-56].

The objective of this study was to develop an LC-MS-MS method to analyze pinacolylmethylphosphonic acid (PMPA; hydrolysis product of soman) and isopropylmethylphosphonic acid (IMPA; hydrolysis product of sarin) from nail clippings. This method provides a complementary long-term matrix to hair for determining nerve agent exposure from terrorist or combat attacks, as well as from long-term occupational exposure.

2. Experimental

2.1. *Materials*

All solvents (HPLC grade or higher) along with formic acid ($\geq 99\%$) were obtained from Fisher Scientific (Hanover Park, IL, USA). Pinacolylmethylphosphonic acid, isopropylmethylphosphonic acid, (PMPA, IMPA; each 1000 $\mu\text{g/mL}$ in methanol), d_7 -IMPA (1000 $\mu\text{g/mL}$ in methanol), and $^{13}\text{C}_6$ -PMPA (100 $\mu\text{g/mL}$ in methanol) were obtained from Cerilliant (Round Rock, TX, USA). Aqueous stock solutions (1 $\mu\text{g/mL}$) were prepared and stored at room temperature. Analytical grade water was acquired at 18.2 $\text{M}\Omega\text{-cm}$ resistivity from a Labconco Water Pro PS water purification system.

2.2. *Nail samples*

Nail samples used for validation experiments were collected from volunteers under the guidelines approved by the Institutional Review Board of South Dakota State University. Volunteers were allowed to collect and submit their own nail clippings. No restrictions were placed on clippings being accepted for the study. No cleaning was performed, except if the nail clippings contained polish, in which case the clippings were rinsed in acetone and allowed to air dry. All samples were stored at room temperature until used. Because of the small amount of sample obtained from each volunteer, clippings from several individuals were pooled during the experiments.

It was not possible to obtain authentic nail samples (i.e., nails containing AMPAs resulting from a nerve agent exposure) to verify the ability of the method presented here to detect AMPAs from the nails of nerve agent exposed animals. Over the duration of the present study, an appropriate animal exposure study could

not be identified mainly due to the cost of housing and caring for the exposed animals for the length of time required to obtain nails which would likely contain AMPAs. Moreover, the number of animal exposure studies that are conducted using authentic nerve agents is extremely limited. Therefore, despite examining multiple studies over 8 years, a suitable study could not be identified. While an animal study was not available to verify the method's applicability to authentic samples, it is common practice in methods related to nerve agent analysis for agents or metabolites to be spiked onto/into biological samples, with no demonstrated analysis from authentic samples [57-63].

2.3. *Sample preparation*

Sample extraction was performed by first adding 50 mg of nail clippings into a 2.0 mL centrifuge tube and spiking the analyte solution (approximately 0.075 mL in each sample) and the internal standard onto the nails. The extraction of AMPAs from nail samples generally followed the extraction protocol of Appel and Logue [ref] for extraction of AMPAs from hair. Briefly, N,N-dimethylformamide (DMF; 0.9 mL) was added to the nails and the tube was capped. The samples were heated for 5 hours on a heat block at 70 °C. They were then shaken for approximately 24 hours (overnight) at room temperature. The DMF was transferred to a 4 mL glass vial and evaporated to dryness under nitrogen. Dried extracts were reconstituted with 0.1% formic acid in a mixture of water and methanol (40:60 water:methanol; 150 µL), syringe-filtered (Millex®-GV, 0.22 µm) into a glass insert (150 µL) contained in a screw-top autosampler vial (2 mL), and analyzed by high performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS).

While hair and nails appear to be very different matrices, they have many similarities. First, both hair and nails are comprised of the protein keratin. Furthermore, metabolites are incorporated in to the hair and nail matrix in the same manner: blood containing the metabolites nourishes the hair follicle or nail root, encapsulating the metabolite as the cells are keratinized. Because of the similar composition and incorporation of compounds from the blood, AMPAs are likely to be extracted from nails in a similar manner as hair. Appel et al. [24] proved AMPAs were extracted from hair using the same procedure outlined here and demonstrated the protective nature of the keratin protein by demonstrating the stability of AMPAs under storage for 5.5 years. The long-term stability of the AMPAs in stored hair offers significant evidence that no appreciable amount AMPAs would be lost during the time it takes for the nail to grow enough to be clipped for analysis.

2.4. Liquid-chromatography tandem mass-spectrometry

The method of Appel et al. [24] was used for the HPLC-MS-MS analysis. Briefly, analysis was performed on a Shimadzu LC system (LC-20AD, Shimadzu Corp., Kyoto, JPN) coupled with a Qtrap 5500 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA). Separation was achieved by reversed-phase (RP) chromatography using a Synergi 4 μ Fusion column (50 mm x 2.00 mm; Phenomenex, Torrance, CA, USA). Mobile phase A consisted of 0.1% formic acid in water, mobile phase B was 0.1% formic acid in methanol, and the injection volume was 10 μ L. The prepared nail extracts were separated with gradient elution at a flow rate of 0.2 mL/min as follows: the column was initially equilibrated with 60% B, linearly increased to 100% B at 4 minutes, maintained at 100% B for 1 minute, and

then decreased linearly back to 60% B over 0.5 minutes, where the column was equilibrated for the next sample (approximately 2 min).

Detection of analytes was achieved by electrospray ionization (ESI) MS-MS, operating in negative ion mode. Nitrogen (20 psi) was used as the curtain gas. The ion source was operated at -4500 V, a temperature of 500 °C, and a pressure of 14 psi for nebulizer (GS1) and heater (GS2) gases. The entrance potential of the collision cell was -10 V. Multiple reaction monitoring (MRM) was used for quantification. One minor modification was made in the MRM transitions for this analysis. The d₇-IMPA quantification transition was changed from 95.0 to 78.9 m/z because the 78.9 m/z provided a better peak shape and slightly greater abundance for quantification. Consequently, 95.0 m/z was designated as an identification transition.

2.5. *Calibration, quantification, and limits of detection*

Nail clippings were spiked to prepare AMPA calibration and quality control (QC) standards as outlined in section 2.3. A ratio of analyte peak area to internal standard peak area was plotted versus AMPA concentration. The linear calibration data for each AMPA was fit by a linear least squares method. Non-weighted and weighted ($1/x$ and $1/x^2$) fits were examined with $1/x$ and $1/x^2$ weighted fits producing the best description of the calibration data for IMPA and PMPA, respectively, as evaluated by the linear range and accuracy of the calibration standards. Data acquisition and peak integration were performed with Analyst™ software, version 1.4.1.

Limits of detection (LOD) were determined by analysis of multiple concentrations of each AMPA. The lowest AMPA concentration that consistently produced a signal-to-noise ratio of 3 was defined as the LOD. Noise was determined as peak-to-peak noise in the baseline of the blank nail over the duration of IMPA or PMPA elution. Lower limits of quantification (LLOQ) and upper limits of quantification (ULOQ) were determined via investigation of calibration standards of IMPA (7.5, 15, 30, 75, 150, 300, 750, 1500, 3000, and 7500 $\mu\text{g/kg}$) and PMPA (0.3, 0.75, 1.5, 3, 7.5, 15, 30, 75, 150, and 300 $\mu\text{g/kg}$) that had a percent relative standard deviation of $<20\%$ (as a measure of precision), as well as an accuracy within $\pm 20\%$ of the nominal concentration when calibration standards were back-calculated from the calibration curve. Three QC standard concentrations (low, medium, and high) for IMPA (50, 200, and 1000 $\mu\text{g/kg}$) and PMPA (2, 20, and 100 $\mu\text{g/kg}$) were prepared in nail clippings, and analyzed in quintuplicate ($N=5$). Fresh QCs were analyzed each day for 3 days along with calibration standards ($N=3$) for inter- and intra-assay investigations.

2.6. *Selectivity, recovery, and stability*

Matrix effects were investigated by creating calibration curves for both aqueous (non-spiked) and spiked nail clippings (all calibration standards were prepared and analyzed in triplicate) and evaluating the slopes of the resulting calibration curves.

Recovery of each AMPA was determined at low, medium, and high QC concentrations by comparing AMPA signals produced from spiked nail clippings and

aqueous samples (N=10). Recovery was calculated as a percentage, relating the peak areas of the spiked nail samples to the peak areas of the aqueous samples.

Storage stability of spiked nail samples was evaluated at high QC concentrations for both IMPA and PMPA at room temperature (N=3). Spiked nail samples were extracted at 0, 3, 6, 12, and 24 hours. After extraction, samples were analyzed together and evaluated relative to the initial analysis (time 0). Autosampler stability was evaluated by placing prepared high QC standards of IMPA and PMPA (N=3) in the LC autosampler, with analysis performed at approximately 0, 2, 4, 8, 12, and 24 hours.

3. Results and discussion

3.1. Analysis of AMPAs by HPLC-MS-MS

Fragmentation of AMPAs by negative ESI-MS-MS analysis was identical to that detailed in Appel et al. [24] Similar fragments were observed for both AMPAs as a result of loss of the side chain from the phosphonyl moiety of the analytes. The parent ions of 179 and 137 m/z represent PMPA and IMPA, respectively ($[M-H]^-$). The MRM transitions of 179 $\rightarrow$ 95 and 179 $\rightarrow$ 79 were used for quantification and identification of PMPA, respectively. Corresponding transitions of 185 $\rightarrow$ 95 (quantification) and 185 $\rightarrow$ 79 (identification) were observed for the labeled internal standard (i.e., the ^{13}C stable isotope labels appear on the pinacolyl group of $^{13}\text{C}_6$ -PMPA). MRM transitions of 137 $\rightarrow$ 79 (quantification) and 137 $\rightarrow$ 95 (identification) were observed for IMPA, as well as the internal standard transitions of 144 $\rightarrow$ 77 (quantification) and 144 $\rightarrow$ 79 (identification).

Figure 3 shows representative chromatograms for the AMPAs from nail samples. The retention times of PMPA and IMPA were similar to those observed in Appel et al. [24], at 4.4 and 3.7 minutes, respectively. The isotopically labeled internal standards (not shown) co-elute with their corresponding non-labeled analyte. The IMPA peak from spiked nail samples was excellent, with a Gaussian peak shape and no significant tailing or fronting observed. Slight tailing can be seen in the trace of the PMPA spiked nails. The chromatography was not changed so as to not compromise the peak shape of the IMPA. Because of the non-ideal PMPA peak shape, integration was performed with extreme care to ensure accurate quantification. The method was very selective, as shown by the lack of other co-eluting chromatographic peaks in the blanks of both analytes. Extraneous, small peaks present in the spiked nail samples (at ~1 minute and after 8 minutes for both AMPAs) are fully resolved from the analyte peaks.

3.2. *Calibration and quantification*

Calibration curves for PMPA and IMPA were prepared using spiked nail clippings. An initial concentration range of 0.3-300 $\mu\text{g/kg}$ was used for PMPA. The calibrators, with concentrations of 0.3 and 300 $\mu\text{g/kg}$, were excluded because they did not meet the linear range inclusion criteria, resulting in a linear range of 0.75-150 $\mu\text{g/kg}$. Similar calibration curves were prepared for IMPA, with concentrations of 7.5-7500 $\mu\text{g/kg}$. For IMPA, the 7.5 and 15 $\mu\text{g/kg}$ calibrators were below the LLOQ, and the 3000 and 7500 $\mu\text{g/kg}$ calibrators were above the ULOQ, resulting in a linear range of 30-1500 $\mu\text{g/kg}$ for IMPA. The slopes of the calibration curves ranged from 0.0982 to 0.1372 and from 0.0862 to 0.1490 for IMPA and PMPA, respectively.

Correlation coefficients (R^2) were from 0.9953 to 0.9980 for IMPA and 0.9966 to 0.9986 for PMPA. The relatively consistent R^2 values and slopes show good linearity and consistency of QC analysis over the three days.

3.3. *LOD, accuracy, and precision*

The LOD for analysis of PMPA and IMPA from spiked nail samples was defined by a signal to noise ratio of 3. The determined LODs for PMPA and IMPA can be found in Table 1. Considering the amount of nail clippings and the volume of solvent used to extract the samples, the LODs correspond to 200 pg/mL and 5 ng/mL for PMPA and IMPA, respectively. Therefore, the method presented here achieved comparable or lower detection limits than many currently available methods.

QC standards at low, medium, and high concentrations were evaluated over 3 days of analysis to assess intra- and inter-assay accuracy and precision. The precision and accuracy were acceptable for both PMPA and IMPA in the concentrations tested, but proved better for IMPA. The intra- and inter-assay precision values for IMPA were <8% and <7%, respectively, while accuracy measurements were within $\pm 12\%$ and $\pm 7\%$ of the nominal QC concentrations for intra- and inter-assay experiments, respectively. PMPA produced a maximum intra- and inter-assay precision of 10%. The accuracy was within $\pm 12\%$ and $\pm 7\%$ of nominal concentrations for intra- and inter-assay studies, respectively. Precision and accuracy were comparable in nails and hair for IMPA. However, both precision and accuracy showed improvement for PMPA in nails as compared to hair.

Table 1. The accuracy, precision, LOD, and recovery of AMPAs from spiked nail clippings.

Analyte	LOD (µg/kg)	LLOQ (µg/kg)	QC Concentration (µg/kg)	Recovery (%)	Intra-assay		Inter-assay	
					Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
IMPA	7.5	30	50	62.2	2.0	100±0.3	6.5	100±5.7
			200	45.0	0.6	100±2.5	5.5	100±6.6
			1000	40.5	4.3	100±4.0	6.2	100±2.4
PMPA	0.3	0.75	2	37.6	6.0	100±1.3	7.1	100±4.4
			20	26.0	6.8	100±5.6	9.8	100±0.6
			100	29.2	10.0	100±7.4	8.9	100±6.6

^aQC method validation (N=5) for day 1^bMean of three different days of QC method validation (N=15)

3.4. Stability, recovery, and matrix effects

Benchtop stability was evaluated at high QC concentrations for both PMPA and IMPA over a 24 hour period. Nail samples were stable at room temperature for up to 12 hours. Not less than 86 and 84% of the original signals were observed through the 12 hour time point for IMPA and PMPA, respectively. At 24 hours, 71 and 79% of the original IMPA and PMPA signals, respectively, were observed. Because these signals fell outside the criteria set for stability (i.e., accuracy within ±20% compared to the signals from the initial sample analysis), the samples were deemed unstable at this time point. Prepared IMPA and PMPA samples were stable in the autosampler for up to 24 hours, with signal variability within 7% and 16%, respectively, when compared to an initial analysis (time 0).

Recovery of PMPA and IMPA was determined by comparing low, medium and high QC concentrations in nail samples with aqueous samples. Recovery of the AMPAs was highly variable among the three concentrations for both AMPAs (Table

1). The average IMPA recovery was 49% (ranging from 40 to 62%), while the average recovery of PMPA was 31% (ranging from 26 to 38%). The variability was likely caused by variations in the pooled nail matrix within each sample which would not exist for a single person. The low recoveries observed were likely due to incomplete extraction of the analytes. Because the extraction is dependent on equilibrium, if the partition coefficient for the AMPAs into DMF from the nails was not large enough for highly efficient extraction, IMPA and PMPA dissolved in the DMF would partition into the nail matrix during the recovery experiment, resulting in a low recovery. While the recoveries were not as high or as precise as hoped, the use of an isotopically labeled internal standard corrects very well for the error associated with the recovery (see Table 1), demonstrating the importance of performing analyses of nail samples using internal standard.

Matrix effects were assessed by comparison of spiked aqueous and nail samples. The slope of the calibration curve made from the nail samples was 25% of the slope of the calibration curve from the aqueous samples. Although not as prevalent, matrix effects were also observed in the analysis of IMPA. When comparing slopes of the IMPA calibration curves, the slope from the nail samples was 38% of that of the aqueous samples. Although matrix effects are significant for the method presented, as with recovery, the internal standard accurately corrects for these effects, as confirmed by the accuracy of the method for both analytes (Table 1).

4. Conclusion

Nail clippings were examined as a matrix for determining past exposure to CWAs by extracting and analyzing PMPA and IMPA. The LC-MS-MS method produced low limits of detection, and excellent precision and accuracy. Although analysis of CWA metabolites from nail clippings via this technique is not appropriate as a universal method to determine exposure (i.e., it takes time for the nail to grow out far enough to be clipped, and typical analysis times are >24 hours), it complements existing methods, and is an alternative method to hair for verification of exposure (Figure 2) when a significant amount of time has elapsed after a suspected exposure.

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Figure 1. Biological reactions of nerve agents and species used to verify exposure. Reaction A indicates the reaction of the nerve agent with the active site on the enzyme (designated R'-OH). Reaction B depicts fluoride reactivation of the enzyme and reformation of the parent agent. Ageing of the agent-enzyme complex is shown in Reaction C. Cleavage of the agent from the enzyme via hydrolysis and direct hydrolysis of the nerve agent are shown in Reactions D and E, respectively.

Figure 2. Longest window of detection, to date, of AMPAs from hair [24], phosphorylated tyrosine [20], reactivated nerve agent [10, 12], inhibited BuChE [19], and AMPAs from urine [8, 23] and blood [23].

Figure 3. Representative chromatograms from the quantification transition for nail samples spiked with PMPA (1.5 mg/kg) and IMPA (15 mg/kg; upper traces) as compared to a non-spiked nail samples (lower traces).