Short communication

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Rapid screening of chemical warfare nerve agent metabolites in urine by atmospheric solids analysis probe-mass spectroscopy (ASAP-MS)

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Exposures to organophosphorus nerve agents (OPNA) remain a threat to both civilian and military populations. Verification of exposures typically involves determinations of urinary metabolites or adducted proteins in blood. Urinary alkyl methylphosphonic acid metabolites resulting from hydrolysis of OPNAs provide a convenient marker for OPNA exposure. In a military setting, urine is a relatively easy sample to obtain, and a rapid turnaround for analyses for the identification of metabolites is critical for field commanders. Timely information on use and identity of OPNAs facilitates decisions regarding employment of personal protective equipment and additional strategies to mitigate additional exposure(s). Herein, we report the development of a rapid mass spectrometric (MS) method to identify OPNA metabolites directly from urine with no sample preparation. Synthetic urine spiked with multiple OPNA metabolites was analyzed using an atmospheric solids analysis probe (ASAP) attached to a high resolution mass spectrometer. The alkyl methylphosphonic acid metabolites resulting from hydrolysis of sarin, cyclosarin, soman, and Russian VX were clearly detectable down to a level of 1.0 ng/ml. The ability to rapidly detect OPNA metabolites in unprepared urine allows for the design of a field-deployable device that could afford field personnel the ability to rapidly screen individuals for specific OPNA exposure. In addition, this provides proof-of-concept evidence that a fieldable ASAP-MS device could afford personnel the ability to rapidly detect OPNAs on skin, equipment, and other porous surfaces. Published 2012. This article is a US Government work and is in the public domain in the USA.

Keywords: organophosphorous nerve agent; acetylcholinesterase

Background

The organophosphorus nerve agents sarin (GB), soman (GD), cyclosarin (GF), VX, and Russian VX (RVX) are extremely toxic compounds due to their relative irreversible binding to the enzyme acetylcholinesterase (AChE). Inhibition of AChE results in excessive accumulation of acetylcholine, which produces hyperstimulation of cholinergic tissues and organs, resulting in a life-threatening crisis in humans.[1] Studies in nerve-agent-exposed animals indicate that OPNAs are rapidly hydrolyzed in the blood to their respective alkyl methylphosphonic acids (AMPAs) (Figure 1) and excreted in the urine.[2] Numerous methods for the analysis of AMPAs in biological fluids, such as plasma and urine, have been developed. These include gas chromatography coupled to mass spectrometry (GC-MS),[3] tandem MS (MS/MS),[4,5] flame photometric detection (FPD)[6,7] or liquid chromatography (LC) with MS/MS.[8,9] The methods require extensive sample preparation prior to analysis, and AMPAs require derivatization prior to GC analysis.

On rare occasions, biomedical samples from individuals exposed to OPNAs have been successfully obtained and analyzed using some of the methods referenced above. The Aum Shinrikyo cult in Japan utilized GB for terrorist attacks in Matsumoto City and the Tokyo subway. Urine samples collected over a seven-day period from one victim of the Matsumoto attack were analyzed for isopropyl methylphosphonic acid (IMPA) and methylphosphonic acid (MPA).[6] The IMPA concentration was 760 ng/ml on the first day after exposure, but had decreased to 10 ng/ml by day seven. Concentrations of MPA were 140 and 20 ng/ml on the first and third day after exposure, respectively.[6] Urine samples from two individuals exposed to GB in the Tokyo attack were also analyzed for the presence of IMPA and MPA. Maximum urine concentrations for IMPA and MPA occurred within 12 and 10–18 h of exposure, respectively.[7]

The purpose of this study was to determine if ASAP-MS could provide adequate sensitivity to detect, not quantify, OPNA urinary biomarkers in unprepared urine to confirm nerve agent exposure and identity in humans. Preparation of biomedical samples prior to analysis is often the most labour-intensive, time-consuming, and error-prone aspect of analysis. Consequently, direct analysis of unprepared urine samples has the potential to provide a rapid screening method as described by

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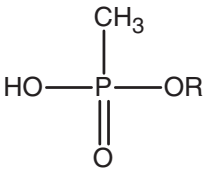

Compound	R	Detected *m/z*
CMPA	C_6H_{11}	179.07991
EMPA	CH_2CH_3	125.02889
IBMPA	$CH_2CH(CH_3)_2$	153.06945
IMPA	$CH(CH_3)_2$	139.05014
PMPA	$CH(CH_3)CH(CH_3)_2$	181.09660

Figure 1. Structures of organophosphorus chemical warfare nerve agent metabolite hydrolysis products found in urine and detected *m/z*. GF (cyclohexyl methylphosphonic acid, CMPA), VX (ethyl methylphosphonic acid, EMPA), RVX (isobutyl hydrogen methylphosphonate, IBMPA), GB (isopropyl methylphosphonic acid, IMPA), and GD (pinacolyl methylphosphonic acid, PMPA).

Twohig and Wilson.[10] The results from these studies indicate that with no sample preparation, sensitivity of the ASAP-MS was better than or comparable to that observed in previously published methods. In principle, the ASAP-MS analysis technique allows for the detection of analytes in a complex mixture based on the analytes volatility, hence the analytes ability to thermally desorb from the sample matrix.

Experimental

An OPNA acid metabolite authentic standard mixture in synthetic urine (proprietary composition) was obtained from Cerilliant (Round Rock, TX, USA). The mixture contained metabolites of GF (cyclohexyl methylphosphonic acid, CMPA), nominal formula weight (FW) of 178 atomic mass units (amu), VX (ethyl methylphosphonic acid, EMPA), nominal FW of 124 amu, RVX (isobutyl hydrogen methylphosphonate, IBMPA), FW of 152 amu, GB (isopropyl methylphosphonic acid, IMPA), nominal FW of 138 amu, and GD (pinacolyl methylphosphonic acid, PMPA), FW of 180 amu all at a concentration of 1.0 milligram per milliliter (mg/ml). Borosilicate melting point (mp) capillary tubes (1.5–1.8 × 100 mm) were acquired from Wilmad LabGlass (Vineland, NJ, USA). The ASAP obtained from M&M Mass Spec Consulting (Hockessin, DE, USA) was fitted to the Ion Max ionization source on an Orbitrap Exactive (Thermo Fisher Scientific, Bremen, Germany) high resolution, high mass accuracy mass spectrometer. The source was modified by fitting the ASAP flange directly into the area designed for photoionization (Figure 2). Nitrogen gas from the evaporation of liquid nitrogen purchased from Airgas (East, Bellmawr, NJ, USA) was used as the heated auxiliary gas for sample vaporization. Heating of the auxiliary gas was accomplished with a heated electrospray (HESI-II) probe on the ion source. The ionization potential was applied through a corona discharge needle inserted into the source opposite the ASAP in a standard source configuration to employ atmospheric pressure chemical ionization (APCI). The Ion Max source and HESI-II probe parameters were ionization mode, positive; sheath gas (N_2), 0 psi; auxiliary gas (N_2), 10 psi (68947.5 Pascal); corona needle, 3.5 kV; capillary temperature, 250 °C, capillary voltage, 77.50 V; tube lens voltage, 60.0 V; heater temperature,

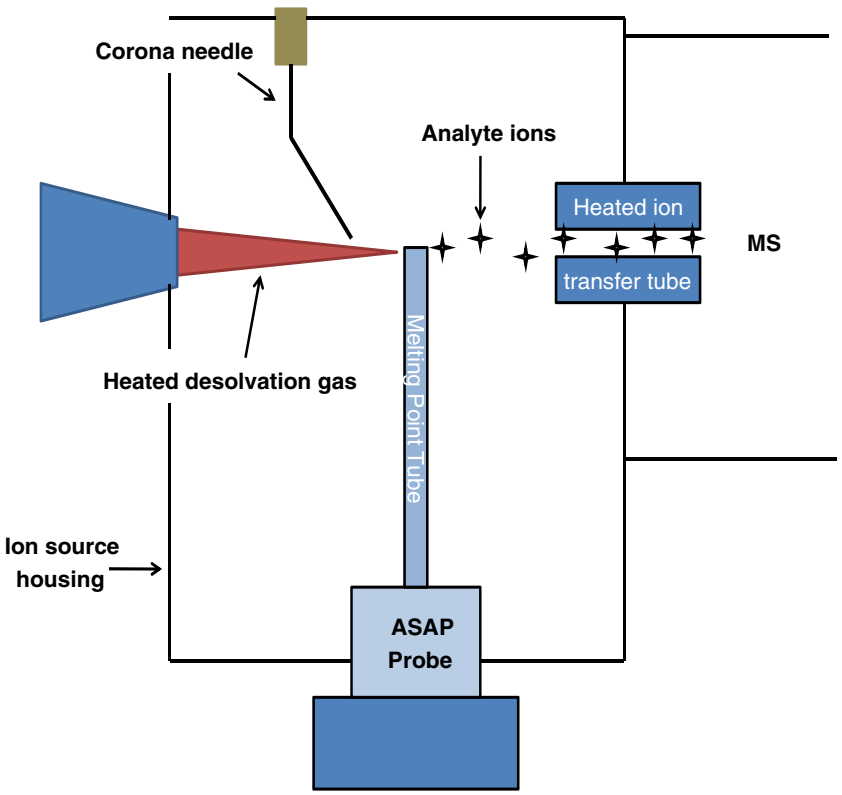

Figure 2. Top view representation of the ASAP aligned in the ion-source housing of the mass spectrometer.

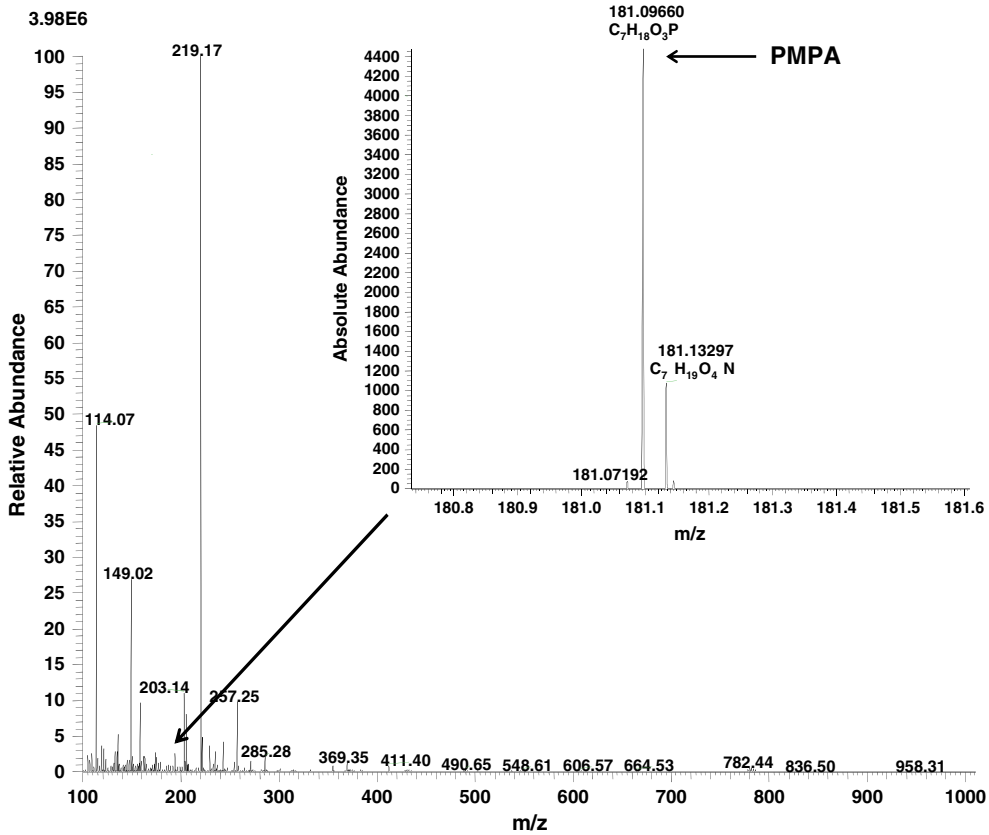

Figure 3. Example of OPNA metabolite spectra in synthetic urine - Shown is ASAP-MS spectra of pinacolyl methylphosphonic acid (PMPA) OPNA metabolite at 1 ng/ml in synthetic urine. Inset spectra is an expanded view of mass range 180.8 to 181.6 showing the protonated molecular ion accurate mass of *m/z* 181.09660 for PMPA. There is no background ion interference from synthetic urine in the PMPA spectra.

ramped from 25 °C to 400 °C after start of data acquisition. Mass spectra were obtained at 100 000 mass resolution (full width at half maximum [FWHM], *m/z* 200).

The sample was applied onto the surface of the mp capillary by dipping the sealed end into the urine standard solution and allowing it to dry at ambient temperature. For analysis, data acquisition was started, the ASAP probe holding the mp capillary was inserted into the ASAP flange attached to the ion source (Figure 2), and the heated auxiliary gas was ramped from 25 to 400 °C. Once 400 °C was obtained, the capillary was rotated within the source to ensure that all analytes of interest were vaporized off the capillary. Total acquisition required about 30 s. Samples were analyzed starting at 100 ng/ml and diluted in half with blank synthetic urine until the detected protonated molecule ($[M+H]^+$) or fragment ion mass fell beyond +/− 5 millimass units (mmu) of the accurate mass confidence level for a metabolite's given $[M+H]^+$ or fragment ion chemical formula. The +/− 5 mmu accurate mass confidence level was set in the Xcalibur QualBrowser (Thermo Fisher Scientific, Bremen, Germany) data acquisition/integration software after instrument calibration.

Discussion

Traditionally, sample matrices containing high salt concentrations are difficult to analyze by GC- or LC-MS and often require clean-up for analysis. As a result, analytes of interest present at low concentrations can be lost, compromising sensitivity of the analysis. The ASAP-MS ionization method described herein offers a technique for direct analysis of OPNA metabolites from high-salt containing sample matrices while eliminating potential analyte loss. In this method, the salts are not thermally desorbed and converted into a gas phase from the glass capillary, primarily because the temperature of the desolvation gas is not adequate for salt desorption. In effect, the separation is a result of the difference in volatility between salts and organic analytes. As a result, only the thermally desorbed and subsequently ionized organic analytes enter the mass spectrometer in the gas phase. This was confirmed by lack of sodium or potassium analyte adduct detection. Additionally, analysis of OPNA urinary metabolites by ASAP removes the need for metabolite derivatization, which is commonly utilized when employing GC techniques for analysis. Lastly, it was found that sodium and potassium salts often present in urine did not produce analyte adducts during ionization because of the relatively low temperature used (400 °C) in sample vaporization relative to that needed to vaporize salts or metals.

Analysis of the OPNA urinary metabolites by ASAP-MS at concentrations of 1.0 ng/ml (CMPA, IBMPA, IMPA, and PMPA) and 100 ng/ml (EMPA) provided accurate *m/z* values within the set 95% accurate mass confidence level. The QualBrowser accurate mass confidence level was set to 95% and was further defined with an elemental composition parameter of only carbon, hydrogen, oxygen, and phosphorus. The calibrated QualBrowser software used the elemental composition criteria to give an accurate mass and molecular formula for the ion(s) detected. In all cases, at 1 ng/ml and above, the mass and molecular formula provided by QualBrowser integration software for metabolites detected was above the 95% confidence level. In

addition, of all the masses and molecular formulas calculated above the 95% confidence level those of the metabolites were the closest to 100% confidence. For samples analyzed with metabolite concentration below 1 ng/ml the QualBrowser integration software did not provide corresponding masses and molecular formulas above the 95% accurate mass confidence level. Although the synthetic urine sample contained CMPA, EMPA, IBMPA, IMPA, and PMPA in equal amounts and all were detected by ASAP-MS, the relative abundances of the $[M+H]^+$ ions for the five metabolites differed. The data suggest that although the compounds have similar structures, enough difference exists such that they all do not ionize equally and therefore have different relative protonated molecular ion abundances. This may be due to the differences in thermal stability of the metabolites. Of the five metabolites analyzed, PMPA produced the most abundant $[M+H]^+$ ion (Figure 3). This may be because PMPA is the most thermally stable followed by IBMPA, IMPA, and CMPA, respectively. Except for CMPA, the ion abundances increased with increasing molecular weight. The molecular weight of CMPA (178 amu) is greater than that of both IBMPA (152 amu) and IMPA (138 amu). The presence of the cycloalkane ring in CMPA may contribute to its thermal instability due to a relative reduction in intra-molecular van der Waals forces. By comparison, IBMPA and IMPA, which have branching alkane chains, would be expected to induce stronger van der Waals forces.

Under the set of conditions employed, the spectra of lower molecular weight metabolites (CMPA, EMPA, IBMPA, IMPA) were not dominated by the $[M+H]^+$ ions, but rather contained a combination of $[M+H]^+$ and fragment ions. Optimal ionization potential/voltage was determined by tuning the mass spectrometers ionization parameters for each analyte to give the best detection/signal for each compounds protonated molecular ion independent of desolvation gas temperature. In addition, the additional ramping of desolvation gas temperature induced fragmentation of the molecular ion. Accurate mass and fragment ions were used to confirm the identity of the individual CWNA metabolites. Detection of the protonated molecular ion was used for confirmation of the analyte and detection of fragment ions from the molecular ion was used as supporting evidence. Protonated molecular ion ($[M+H]^+$) abundance alone did not determine the relevance of this analytical technique as a quick screening method for OPNAs in urine. Under these ionization conditions, relative abundance of protonated molecular or fragment ions merely reflects the analytes' propensity to thermally fragment, or not.

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