Introduction

Determining the use of chemical warfare agents (CWAs) in times of war or in acts of terrorism requires methods for the rapid analysis of biological samples (urine and blood) to confirm exposure. In the event a large number of people are exposed to a CWA, screening analysis of the environmental and biomedical samples may determine the specific agent of exposure. Although health professionals will treat the symptoms of casualties, a more effective treatment can be provided if the chemical causing the symptoms is known. Furthermore, the rapid analysis of metabolites would help health care providers to distinguish between people who were exposed and people experiencing a panic attack.

Short-lived metabolites of CWAs are eliminated by the body, usually via the urine, and can only be detected in a period of 24 h to several days post-exposure, while long-lived metabolites can be found in the urine or blood for days to several weeks after exposure (1). In cases of alleged CWA use, particularly in remote conflicts, samples are more likely to be collected several days or even weeks after the event (2). For sulfur mustard exposure, long-lived metabolites include 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTESE) and 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE), nucleic acid and protein adducts (3). For nerve agents, alkyl-phosphonic acids are specific biomarkers (4).

Nerve agents and sulfur mustard metabolites can show up quickly in urine, the traditional biological fluid for testing, because urine sample collection is not invasive. Disadvantages of urine are its inherent variability and complex composition. Therefore, such challenging matrix such as urine requires the use of liquid chromatography–tandem mass-spectrometry (LC–MS–MS) approaches, which can overcome some of the problems by using a highly selective and sensitive ion monitoring technique. In addition, because the CWA metabolites are polar organic compounds, the use of LC–MS–MS negates the need for derivatization techniques required by gas chromatography–mass spectrometry (GC–MS) analysis methods.

Several researchers have used LC–MS techniques for the analysis of drugs of abuse and toxic compounds in urine (3–8). Labor-intensive sample preparation techniques described have included simple filtration, protein precipitation with ultrafiltration and automated solid phase extraction. In cases of sublethal and lethal poisoning during Tokyo sarin disaster, the observed concentrations of analytes in urine were at sub-μg/mL concentration (8). Validated quantitative methods have demonstrated limits of detection (LODs) at sub-ng/mL concentration (4, 7). To our knowledge, no fully validated 'dilute-and-shoot' LC–ESI–MS–MS analysis method for these compounds in urine is described in the scientific literature. The purpose of this study was to demonstrate the feasibility of direct injection of non-extracted non-derivatized diluted urine samples for the sensitive monitoring of several CWA metabolites using the fast liquid chromatography–tandem mass-spectrometry (RSLC–MS–MS) approach.

Materials and methods

Chemicals

Acetonitrile (gradient grade) was purchased from Panreac (Barcelona, Spain) and deionized water was from Milli-Q system (Millipore, USA). Formic acid was obtained from Sigma Chemical (St Louis, USA). Methanol was purchased from Burdick and Jackson (Seelze, Germany). Ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (iPrMPA) and pinacolyl methylphosphonic acid (PMPA) were purchased from Cerilliant (Austin, TX). Isobutyl methylphosphonic acid (iBuMPA) was synthesized in-house. MSMTESE and SBMSE were made available by the Organization for the Prohibition of Chemical Weapons (OPCW) in the Biomedical Confidence Building Exercise as reference standards in water with a concentration of 10 μg/mL.

Preparation of standard solutions, quality controls and samples

An aqueous solution of the analyte standards was prepared by dilution to the concentrations of 1, 5, 20, 50 and 100 ng/mL. Quality control (QC) intermediate standards were prepared at the concentrations of 10, 40 and 80 ng/mL of each analyte. Short-term storage of the fortifying solutions was at 4°C. Long-term storage (>3 days) was at −20°C.

Urine samples from anonymous donors were collected in prescreened cups. The matrix-matched alkyl methylphosphonic...
acids, SBMSE and MSMTESE standards and QCs were created by fortifying 500 μL of healthy donor urine with aliquots of 10 μg/mL alkyl methylphosphonic acids, SBMSE and MSMTESE standards and pure water for a total volume of 1 mL so that the final standards concentrations of analytes in urine were 1, 5, 20, 50 and 100 ng/mL with QC concentrations of 10, 40 and 80 ng/mL. A blank QC was the urine sample without alkyl methylphosphonic acids, SBMSE and MSMTESE.

**Urine sample pretreatment**

In this process, 0.5 mL of deionized water and 0.5 mL of blank or fortified urinary standard were mixed and transferred to LC autosampler vials for injection into the LC–MS-MS instrument.

**Liquid chromatography mass spectrometry**

Analytical separations were performed with a Dionex Ultimate 3000 liquid chromatograph equipped with a 150 mm × 2.1 mm, 2.2 μm Acclaim 120 C18 column (Dionex, USA) at 30°C. Twenty microliter injections of the samples and standards were made three times on the RSLC using a mobile phase consisting of 0.5% formic acid in water (Solvent A) and 0.5% formic acid in acetonitrile (Solvent B). Separation was performed by gradient elution applying the following gradient program: 98% A and 2% B at a flow rate of 0.45 mL/min was held for 1.5 min. Between 1.5 and 8 min, the mobile phase changed to 100% B. After that, the gradient turned back to 98% A during 0.3 min which was held for 4 min for equilibration. The compounds were analyzed by electrospray/tandem mass spectrometry operating in multiple reaction monitoring (MRM) mode on an 4000 QTRAP® LC–MS-MS system (AB Sciex, Canada). For the MS, the following conditions were used: ion spray voltage, ±4500 V (negative ion mode for alkyl methylphosphonic acids detection and positive ion mode for SBMSE and MSMTESE detection); ion source heater temperature, 350°C; ion source gas (N2) for nebulizing, 0.21 MPa; ion source gas (N2) for drying solvent, 0.28 MPa; curtain gas (N2), 103.42 kPa. Specific individual compound parameters (i.e., declustering potentials (DP) and collision energies (CE)) were optimized for each analyte. Dwell time was set at 50 ms for each product ion.

![Figure 1](https://academic.oup.com/jat/article-abstract/39/1/69/2798059)
Method validation

Selectivity
Six different blank urine samples were analyzed to investigate the possible interference of endogenous compounds.

Precision and accuracy
To determine the interday precision five replicates of each QC sample were analyzed on the same day. The intraday precision was established in the same way on four different days. To determine the accuracy, for each QC sample the calculated concentration was compared with the theoretical concentration.

Matrix effects were assessed using replicates (n = 5) of QC samples. The matrix effects were investigated by comparing fortified urine blank samples with standard solutions of analytes.

Stability
Short-term stability was determined by analyzing QC samples kept at room temperature for 12 h. Samples were analyzed every 4 h. To investigate long-term stability, the samples were kept at −20 °C for 10 days. The samples were analyzed after 2, 5 and 10 days of storage. Freeze–thaw stability was determined by analyzing the samples undergoing three freeze (−20 °C)–thaw (room temperature) cycles.

Figure 2. The MRM ion chromatograms of human urine samples after ‘dilute-and-shoot’ procedures: (a) fortified with 5 ng/mL of each alkyl-phosphonic acids, (c) non-fortified urine, alkyl-phosphonic acids MRM mode detection; (b) fortified with 5 ng/mL of each SBMSE and MSMTESE and (d) non-fortified urine, SBMSE and MSMTESE MRMs mode detection.

Table I
<table>
<thead>
<tr>
<th>Compound</th>
<th>Corresponding agent</th>
<th>Precursor ion, m/z</th>
<th>Product ion, m/z</th>
<th>DP (V)</th>
<th>EP (V)</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBMSE</td>
<td>HD</td>
<td>247</td>
<td>163</td>
<td>26</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>MSMTESE</td>
<td>HD</td>
<td>231</td>
<td>167</td>
<td>25</td>
<td>9</td>
<td>29</td>
</tr>
<tr>
<td>EMPA</td>
<td>VX</td>
<td>−123</td>
<td>−95</td>
<td>−18</td>
<td>−8</td>
<td>−14</td>
</tr>
<tr>
<td>iPMPA</td>
<td>GB</td>
<td>−137</td>
<td>−95</td>
<td>−22</td>
<td>−8</td>
<td>−16</td>
</tr>
<tr>
<td>iBuMPA</td>
<td>RVX</td>
<td>−151</td>
<td>−95</td>
<td>−28</td>
<td>−10</td>
<td>−21</td>
</tr>
<tr>
<td>PMPA</td>
<td>GD</td>
<td>−179</td>
<td>−95</td>
<td>−29</td>
<td>−10</td>
<td>−21</td>
</tr>
</tbody>
</table>

Dilute-and-Shoot RSLC–MS/MS Method
Data analysis

Peaks representing the alkyl methylphosphonic acids, SBMSE and MSMTESE were integrated by reprocessing the data with Analyst 1.5 software to obtain peak-area ratios. To evaluate the linearity of the analytical method, the fortified urine calibration standards were prepared and analyzed in duplicate on five different analytical runs. Quantitation was performed using the peak area of each analyte. Weighted (1/y²) least-square linear regressions were used to obtain the equation of the calibration curves. The LODs for analytes were calculated with a signal-to-noise of 3:1 (Figure 1). This product ion was used for quantitation of all four nerve agent metabolites. The m/z [M-H]⁻ → 79 confirmation ion transition was chosen for EMPA and iPrMPA (DP = −20 V, CE = −15 V) and also for iBuMPA and PMPA (DP = −29 V, CE = −21 V).

SBMSE and MSMTESE undergo cleavage of the methylsulfinyl group giving fragments at m/z 183 and 167, respectively (Figure 1). The MRM experiments were performed for the quantitation ion (m/z 247 → 183), and the confirmation ion (m/z 247 → 119) transitions of SBMSE (DP = +26 V, CE = +12 V). Chosen quantitation ion (m/z 231 → 75) and the confirmation ion (m/z 231 → 167) transitions of MSMTESE had the highest intensity when the DP value was +25 V, and the CE value was +29 V.

The % relative standard deviation (RSD) for retention times obtained from three consecutive analyses of 50 ng/mL solutions for organophosphorus nerve agent metabolites, MSMTESE and SBMSE, in urine were in the range of 0.49–2.70%. In addition to the selective detection of diagnostic product ions obtained by the simultaneous MRM measurement in electrospray ionization-tandem mass-spectrometry (ESI–MS-MS), the separations of alkyl methylphosphonic acids, SBMSE and MSMTESE by this procedure enabled us to perform reliable qualitative analysis in unknown samples such as human urine.

Validation

Selectivity

No interfering peaks from the endogenous urine compounds were observed at the retention times of each analyte. The representative chromatograms of the blank urine sample and fortified urine are shown in Figure 2a and b.

Linearity

The calibration curves of alkyl methylphosphonic acids, SBMSE and MSMTESE in human urine over a 3-week period were analyzed. RSLC–MS-MS analysis, using MRM mode, was used to determine LODs and the linear dynamic ranges of alkyl methylphosphonic acids, SBMSE and MSMTESE.

The calibration curves for analytes were plotted over a concentration range of 1–100 ng/mL with two injections at each concentration (correlation coefficients >0.997 for urine samples fortified with alkyl methylphosphonic acids, SBMSE and MSMTESE).

Each individual calibration curve showed excellent linearity with correlation coefficients generally ≥0.99. The calibration curve spanned two orders of magnitude with a linear working

Results and discussion

RSLC–MS-MS conditions

Utilization of RSLC–MS-MS for the quantitation of organophosphorus nerve agent metabolites, MSMTESE and SBMSE, demonstrated approach sensitivity, selectivity and robustness. The compounds ionized readily under electrospray conditions, chromatographically peaks were chromatographically well resolved and the product ions from collision-induced dissociation are distinct (Figure 1). Complete resolution between the peaks of nerve agent metabolites. The m/z 247 → 183, and the product ions from collision-induced dissociation are displayed (Figure 1). This product ion was used for quantitation of all four nerve agent metabolites. The m/z [M-H]⁻ → 79 confirmation ion transition was chosen for EMPA and iPrMPA (DP = −20 V, CE = −15 V) and also for iBuMPA and PMPA (DP = −29 V, CE = −21 V).

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Each individual calibration curve showed excellent linearity with correlation coefficients generally ≥0.99. The calibration curve spanned two orders of magnitude with a linear working
range from the lowest to the highest standard. The standard deviations show the excellent reproducibility of repeated measurements at each standard concentration.

**Limit of detection**
The LODs were calculated as $3 \sigma_0$, where $\sigma_0$ is the standard deviation value when the concentration approaches zero. We
determined $s_0$ from the replicate analysis of low concentration standards (i.e., 1 ng/mL). Corresponding LODs (signal/noise ≥ 3/1) were established as 0.5 ng/mL. The limiting factor in overall sensitivity was found to be the decreased signal intensities of EMPA, SBMSE and MSMTESE in comparison with the other analytes in the list. However, an urinary LOD of 0.5 ng/mL was observed for each analyte during the experiment with fortified urine (Table I) and for the iPrMPA, iBuMPA and PMPA would be much lower than 0.5 ng/mL based on a 3-to-1 signal-to-noise ratio.

**Accuracy and precision**

As a part of the validation process, QC samples were analyzed in 20 repeated measurements. QC was found to be acceptable at all three concentrations as exemplified in the plot of data from the lowest concentration QC in Figure 3. The average low, medium and high QC results for EMPA (9.7, 43 and 77 ng/mL, respectively) were in good agreement with the calculated concentrations of 10, 40 and 80 ng/mL. RSDs for alkyl methylphosphonic acids, SBMSE and MSMTESE for the low QC samples were <11%, for the medium QC samples they were <7% and for the high QC samples they were <6%. The data for intra- and interday precision and accuracy are presented in Table II. With regard to matrix effects, all concentrations were between 95% and 105% of the nominal values (Table II), suggesting that there were no significant matrix effects for alkyl methylphosphonic acids SBMSE and MSMTESE and that no co-eluting substance influenced the ionization of the analytes. The results were reproducible and intra- and interday precisions were <9%. The accuracy values were in the range 96.0–108.0%. The accuracy and reproducibility of the fortified human urine samples demonstrate the applicability of this method in a diverse range of urine matrices and concentrations.

**Stability**

The results demonstrated the reliable stability behavior of alkyl methylphosphonic acids, SBMSE and MSMTESE under tested conditions. The obtained data are summarized in Table III. No significant difference (<5%) was observed between alkyl methylphosphonic acids, SBMSE and MSMTESE concentrations at zero time and at the end of the experiments.

**Application to the OPCW biomedical confidence exercise**

Developed ‘dilute-and-shoot’ RSLC–MS-MS method was used in the OPCW Second Biomedical Confidence Building Exercise (Figure 4) by the Laboratory for the Chemical and Analytical Control of Military Research Centre. Reasonably, one of the major tasks of the test was correct identification of CWA biomarker in urine samples. Quantitation reported by some laboratories was generally close to the nominal fortifying concentrations. Those methods that used isotopically labeled internal standards and calibration in fortified urine generally gave the better results. Our results are listed in Table IV.

**Conclusion**

The results suggest that the ‘dilute-and-shoot’ RSLC–MS-MS method has potential for the rapid determination of exposure to sulfur mustard and nerve agents from urine. The detection limits obtained are at or below the background concentrations for the target analytes. The analysis of urine samples to determine exposure to sulfur mustard and nerve-type agents is feasible. The advantages of this technique are that urine is more easily obtained than blood, and the sample preparation steps are minimal. This novel ‘dilute-and-shoot’ approach can be used for monitoring purposes followed by more sensitive isotope dilution solid phase extraction liquid chromatography tandem mass-spectrometry (SPE–LC–MS-MS) analysis to obtain accurate values for excretion curves or to obtain dose-response relationships for the different CWAs. The approval of the approach was made during the OPCW Second Biomedical Confidence Building Exercise.

**Table IV**

<table>
<thead>
<tr>
<th>Sample description Chemical</th>
<th>Retention time, min</th>
<th>Regular amount, ng/mL</th>
<th>Determined concentration, ng/mL$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A-15/3 EMPA</td>
<td>1.9</td>
<td>25</td>
<td>18.8</td>
</tr>
<tr>
<td>Sample B-15/4 EMPA</td>
<td>2.0</td>
<td>25</td>
<td>13.5</td>
</tr>
<tr>
<td>Sample C-15/4 iPrMPA</td>
<td>5.6</td>
<td>25</td>
<td>14.9</td>
</tr>
<tr>
<td>Sample D-15/4 iPrMPA</td>
<td>3.9</td>
<td>5</td>
<td>5.5</td>
</tr>
<tr>
<td>Sample E-15/4 SBMSE</td>
<td>1.9</td>
<td>25</td>
<td>10.0</td>
</tr>
</tbody>
</table>

$^*$The evaluation for each compound was performed using only one reference methanol standard (10 ng/mL).