Evaluation of a Procedure for the Simultaneous Quantification of 4-Ketocyclophosphamide, Cyclophosphamide, and Ifosfamide in Human Urine

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Abstract

An accurate and precise analysis procedure is presented for the detection and quantification of cyclophosphamide (CP), 4-ketocyclophosphamide (4-keto-CP), a primary metabolite of CP, and ifosfamide (IF) in human urine. CP and IF are common antineoplastic drugs used for the treatment of many types of cancer. Workers in the healthcare field, including nurses and pharmacists who interact with or prepare prescriptions for patients, have potential low-level exposure to the parent drugs; therefore, an analysis procedure is needed. The main focus of this procedure is the quantitation of 4-keto-CP because it is a primary metabolite of CP exposure and stable under physiological conditions. Sample preparation consists of liquid–liquid extraction of urine with ethyl acetate, and the analysis consists of reversed-phase high-performance liquid chromatography coupled with tandem mass spectrometry for detection of the analytes. Accuracy and precision of this procedure is demonstrated by means of recovery experiments. Recoveries are between 97–105% of theory for the three target analytes at various concentrations (25, 50, 100, and 375 ng/mL for 4-keto-CP; 1, 2, 4, and 15 ng/mL for CP and IF) with relative standard deviations of 8.4% or less. The limit of detection for this procedure is 1 ng/mL for 4-keto-CP, 0.1 ng/mL for CP, and 0.05 ng/mL for IF in urine.

Introduction

Cyclophosphamide (CP) or 2-(bis(2-chloroethyl)amino)tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide and ifosfamide (IF) or (2-chloroethylamino)-N-(2-chloroethyl)-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide (Figure 1) are two of the most widely used antineoplastic alkylating agents (1,2). Both compounds are utilized in chemotherapy because of their activity against a variety of human tumors (1,2). Carcinogenic risks are related to secondary malignancies in patients under CP or IF treatment as well as possible occupational hazards for healthcare workers who handle these compounds in oncologic medical units. Concerns have increased in recent years about the occupational health risks to pharmacists who prepare these drugs and to nurses who administer these drugs, handle patient excreta, or clean contaminated medical equipment (3,4).

CP is a pro-drug and requires metabolic activation to phosphoramid mustard, the cytotoxic antineoplastic agent. This metabolic activation initially starts in the liver by means of cytochrome P-450. The predominant pathway of CP metabolism includes the formation of 4-hydroxycyclophosphamide (4-OH-CP), which either tautomerizes to form aldophosphamide (AP) or is further oxidized to 4-ketocyclophosphamide (4-keto-CP), an inactive compound shown in Figure 1. AP undergoes non-enzymatic β-elimination to generate acrolein and phosphoramide mustard. Additionally, the AP metabolism yields the inactive carboxycyclophosphamide (carboxy-CP) (5). IF, a structural analog of CP, is activated by the formation of ifosfamide mustard by the same metabolic route. The effectiveness of IF is complicated by the presence of a major pathway, dechloroethylation, which results in the formation of 2- or 3-dechloroethylifosfamide and chloroacetamide, a nephrotoxicant (6). Phosphoramide mustard and ifosfamide mustard both can alkylate DNA (5,7). The antineoplastic effects of CP and IF are not specific for tumor cells but are more toxic to rapidly proliferating cells.

Figure 1. The chemical structures of (A) cyclophosphamide, (B) 4-ketocyclophosphamide, (C) ifosfamide, and (D) CP-d6.
Most recent occupational field studies have only found trace levels of CP (7–9) or IF, if at all. It was the desire of this study to develop an analytical procedure to detect and quantify the most likely metabolite-based biomarkers for these drugs, such as 4-keto-CP as well as the parent compounds. Many of the metabolites of CP and IF are not stable in blood or urine. For example, carboxy-CP and carboxylosamidase have been reported to be not stable in urine even in cold storage at –80°C (10); such unstable metabolites would be poor choices as possible biomarkers for exposure assessment of workers. Numerous methods, such as gas chromatographic (GC) and high-performance liquid chromatographic (HPLC), have been developed to detect and quantify either CP and/or IF in biological matrices (7–9,11–22). GC methods have required derivatization of the CP and IF to volatilize the analytes, while the HPLC methods have usually not required that extra step in sample preparation. CP is not thermally stable at temperatures above 200°C (19), which can create problems for GC-based methods. Therefore, HPLC-based methods have a clear advantage, and HPLC was chosen for this work. Also, the use of tandem mass spectrometric detection (MS–MS) with HPLC would add an important level of method specificity as well as increased sensitivity.

Most of the analysis methods in the literature have been used for clinical dose levels, or they have focused on a single analyte such as CP and use IF as an internal standard. Clinical dose methods are based on high levels of drugs and not the low exposure levels typically involved in occupational exposure, which would require the detection of trace levels of either the parent drug or its metabolites. Although several methods have been developed for trace level analysis to cover possible exposure by healthcare workers of the parent drugs (7,11,16–18,20,21), it was the desire of this laboratory to develop a method capable of detecting and quantifying the likely CP stable metabolite, 4-keto-CP, as well as the parent compound in urine. The need for obtaining improved sensitivity often requires more tedious sample preparation and sample pre-concentration within a test method. Also, because this method was mainly focused on the quantification of 4-keto-CP, a detection limit less than the 5 ng/mL previously reported in the literature (14) was desired. Another objective was to develop a simple and effective extraction procedure along with HPLC–MS–MS conditions to enable the simultaneous determination of 4-keto-CP, CP, and IF at trace levels in human urine. In turn, this method could be used to support field studies of healthcare workers who have possibly been exposed to these compounds. It was also our goal to have a fully validated test method (23,24) for current and future field studies.

**Experimental**

**Chemicals and reagents**

The 4-keto-CP was obtained from Niomech (Bielefeld, Germany). CP monohydrate reference standard was purchased from Sigma-Aldrich (St. Louis, MO), and IF reference standard was purchased from American Pharmaceutical Partners (Schaumburg, IL). The deuterated CP analog (CP-d6) was obtained from CPS Chemie + Service (Duren, Germany). Acetonitrile and HPLC-grade water were obtained from Burdick & Jackson (Honeywell International, Muskegon, MI). Water for all sample preparation was obtained from a Barnstead NANOpure system (Thermo Fisher Scientific, Pittsburgh, PA). All other reagents used were analytical-grade and are regularly available in a laboratory.

**Instrumentation and chromatographic conditions**

The chromatographic analysis was carried out using an Agilent Technologies model 1100 liquid chromatograph (Palo Alto, CA) with pump, degasser, and cooled autosampler (7°C), and it was equipped with an Agilent model 6410A triple quadrupole MS–MS with an electrospray ionization (ESI) interface. The analytical column used was an Agilent Zorbax RX C18 (250 × 3 mm i.d., 3.5 µm). A Phenomenex C18 SecurityGuard guard cartridge (4 × 2 mm) (Torrance, CA) was used in-line ahead of the analytical column. The column temperature was set at 25°C, and gradient elution at a constant flow rate of 0.3 mL/min was used. Mobile phase A contained acetonitrile–water–acetic acid (15/85/0.1%, v/v/v), and mobile phase B contained acetonitrile–water–acetic acid (75/25/0.1%, v/v/v). For each chromatographic run, the mobile phase composition was held initially for 2 min at 100% A, then a linear gradient proceeded to 30% B over a 10-min interval. A second linear gradient proceeded from 30% B to 100% B over an 8-min interval. A hold at 100% mobile phase B for 7 min was used to remove any well-retained urine sample components from the column; then, the system was re-equilibrated back to initial conditions with mobile phase A. A 10-µL volume of chromatographic sample was injected for LC–MS analysis, and data was acquired for roughly 25 min after injection. The electrospray source was operated in the positive ion mode. The electrospray voltage was set at 3000 V. The nebulizer gas flow was 10 L/min at a pressure of 35 psi and a temperature of 325°C. Dwell time was 200 ms, fragmentor voltage was 140 V, and collision energy was set at 25 V for all masses. The collision gas was nitrogen, and it was set at the factory default flow rate of 0.06 L/min. The mass transitions monitored for quantification of the analytes were m/z 261 → 140 for CP, m/z 261 → 154 for IF, m/z 275 → 106 for 4-keto-CP, and m/z 267 → 140 for CP-d6. Mass transitions for quantification of the analytes were also monitored; these consisted of m/z 261 → 106 for CP, m/z 261 → 92 for IF, and m/z 275 → 204 for 4-keto-CP.

**General urine sample preparation**

Non-fortified urine samples or analyte fortified urine were treated identically. A 4.0-mL portion of the urine was placed in a screw-capped tube, and 0.5 mL of 2 M potassium phosphate buffer (pH 7.0) was added. A 0.25-mL aliquot of a 50 ng/mL CP-d6 internal standard solution was added. A 0.25-mL portion of deionized water for test sample urine or analyte-spiking solution was added to the fortified urine samples. The urine sample was extracted three times with 5.0 mL of ethyl acetate using a vortex mixer for 1 min for each extraction. The ethyl acetate layers were volatilized, while the HPLC methods have usually not required that extra step in sample preparation. CP is not thermally stable at temperatures above 200°C (19), which can create problems for GC-based methods. Therefore, HPLC-based methods have a clear advantage, and HPLC was chosen for this work. Also, the use of tandem mass spectrometric detection (MS–MS) with HPLC would add an important level of method specificity as well as increased sensitivity.

Most of the analysis methods in the literature have been used for clinical dose levels, or they have focused on a single analyte such as CP and use IF as an internal standard. Clinical dose methods are based on high levels of drugs and not the low exposure levels typically involved in occupational exposure, which would require the detection of trace levels of either the parent drug or its metabolites. Although several methods have been developed for trace level analysis to cover possible exposure by healthcare workers of the parent drugs (7,11,16–18,20,21), it was the desire of this laboratory to develop a method capable of detecting and quantifying the likely CP stable metabolite, 4-keto-CP, as well as the parent compound in urine. The need for obtaining improved sensitivity often requires more tedious sample preparation and sample pre-concentration within a test method. Also, because this method was mainly focused on the quantification of 4-keto-CP, a detection limit less than the 5 ng/mL previously reported in the literature (14) was desired. Another objective was to develop a simple and effective extraction procedure along with HPLC–MS–MS conditions to enable the simultaneous determination of 4-keto-CP, CP, and IF at trace levels in human urine. In turn, this method could be used to support field studies of healthcare workers who have possibly been exposed to these compounds. It was also our goal to have a fully validated test method (23,24) for current and future field studies.
Standard sample preparation and recovery studies

CP and IF standards for calibration curves were prepared at the 0.5, 1, 2, 10, 20, and 25 ng/mL equivalent levels in urine plus a blank 0 ng/mL level sample. 4-Keto-CP had a lower sensitivity than CP or IF, so standards for its calibration curve were prepared at the 10, 12.5, 25, 50, 250, 500, and 625 ng/mL equivalent levels in urine. Blank urine, from a common source known to contain none of the analytes, was used to make fortified samples at 1, 4, and 15 ng/mL CP and IF levels and 25, 100, and 375 ng/mL 4-keto-CP levels for each trial batch of the primary recovery experiment.

A secondary recovery experiment consisted of collecting urine from 20 non-exposed volunteers and preparing them as individual samples. Urine samples containing no analytes or internal standard, urine samples spiked with CP-d₆ only, and urine samples spiked with 2 ng/mL CP and IF and 50 ng/mL of 4-keto-CP along with the internal standard were prepared for this second recovery experiment.

Calculations

All analyte quantification for the validation of this test procedure was based on peak-area ratios of the three analytes to CP-d₆ for recovery studies. The initial extraction efficiency studies were based on peak areas of the extracted sample to those of known standard solutions. The standard calibration curves used for the recovery studies were linear within the 0.5–25 ng/mL CP and IF range; correlation coefficients were 0.99 or greater, and y-intercepts approached zero for all curves generated with this procedure. Linearity was also verified for 4-keto-CP response. Within the 10–625 ng/mL concentration range, correlation coefficients were 0.99 or greater, and y-intercepts approached zero for all curves generated during this procedure’s validation. Calibration curves were generated at the beginning, middle, and end of each recovery study chromatographic run to verify the lack of any significant calibration curve drift during the use of this method. Sample quantification was calculated using the bracketed standard curves; the preceding and following curves were used, and the two assay values were averaged.

The limit of detection (LOD) was calculated in a traditional manner (23,24) using three times the noise level divided by the slope of the calibration curve. Because instrumental noise is a function of height, the average baseline level of height noise was determined for each batch run in chromatograms at the retention time window for each analyte from the blank samples. This was done by exporting raw data files into Microsoft Excel and determining the mean height level and the standard deviation of height noise from 100 data points within the retention time window noted for specific analyte monitoring specific transition signal. The slope from the calibration curve using peak heights of all the standard solutions was determined and then used as the divisor for this LOD calculation. It should be noted that peak heights was used only for the estimation of the LOD; peak areas or peak-area ratios were used for quantification of the analytes during the validation of this procedure.

Results and Discussion

Chromatography and detection

The optimized chromatographic conditions developed for this procedure proved to be specific and have no major interferences and enabled the simultaneous quantification of the three target analytes. All non-fortified urine samples chromatographed showed no interfering peaks. The blank samples from 20 non-exposed volunteers showed no interferences for CP, IF, 4-keto-CP, or the CP-d₆ internal standard at the selected mass transitions.
used for quantification. A typical chromatogram is shown in Figure 2A, which shows a signal plot from a fortified urine sample containing 2 ng/mL equivalent level of CP and IF, a 50 ng/mL level of 4-keto-CP, and a 3.1 ng/mL level of the internal standard (broken line). The chromatographic baselines displayed little drift from the gradient run with the exception of the CP signal, but smaller background peaks did not interfere with the three analytes of interest. In the case of m/z 261 → 140 for CP, the baseline drift and noise was less significant after approximately 10 min into the gradient program. Because CP eluted at approximately 14 min, a reasonable baseline was obtained during its retention time window. Figure 2B–2C display chromatograms of the standard urine spiked at its lowest level (B) and a non-fortified urine sample with no analyte spikes (C). The urine used for the chromatograms displayed in Figures 2B–2C were from the same common source, which had no trace level of any of the analytes or the internal standard. Again, it is clearly shown that none of the background urine peaks interfere with the analyte peaks or the deuterated internal standard. Figure 2D shows a urine sample containing only the CP-d₄ internal standard, which does not interfere with the CP trace as would be expected of the triple quadrupole mass spectrometer. Sample carry-over was eliminated in this procedure by the use of a (50/50, v/v) acetonitrile–water rinse of the autosampler injector needle and loop. This was found to be necessary during this procedure’s early development because CP apparently did produce a small peak near the LOD when not using the rinse. When using the rinse, no CP was detected in blank urine preparation injected after the highest standard level.

The MS analysis of CP and IF has been described in detail by Liu et al. (25). The mass transitions chosen to detect CP and IF used in this procedure were those that had the greatest response (Figure 4). Because this procedure was designed for use as a test for biomarkers of exposure, high sensitivity was desired (26); thus, the choice of transitions that yielded higher detector responses was required. The mass transitions monitored for quantification of the analytes were m/z 261 → 140 for CP and m/z 261 → 154 for IF. For CP, m/z 261 → 140 gave the largest response. For IF, m/z 261 → 154 gave the best signal-to-noise ratio. The mass transition for 4-keto-CP, m/z 275 → 106 (Figure 3), was chosen because it was more unique for the compound compared to other possible metabolites of IF, which had similar chromatographic retention times. The m/z 275 → 106 was confirmed by scanning parent ion m/z 277 and finding the m/z 106 to m/z 108 ratio correct for the chlorine isotope ratio. Also, the work by Liu et al. (25) generally showed that the ring structure of CP, IF, and their related metabolites did not form stable ions during fragmentation.

The qualifying mass transitions for CP and IF were selected as the second highest response that did not have significant interferences from components existing in urine. This is a generally accepted practice (27). The qualifying mass transition for 4-keto-CP was chosen for its unique signal for the compound; the corresponding metabolite for IF would have a similar chromatographic retention time, so a unique ion was again necessary. Mass transitions for qualification of the analytes were also monitored for assurance of peak identity, although no interferences were discovered during this procedure’s development. The qualifying mass transition signals were valid for use at the lowest standard concentration for each analyte, which is appropriate for the lower limit of quantification (LLOQ) defined by the U.S. FDA (28), and thus, were considered adequate for this test procedure.

The LOD for this procedure was calculated to be 1 ng/mL for 4-keto-CP, 0.1 ng/mL for CP, and 0.05 ng/mL for IF in urine. In an HPLC–MS method for the detection of CP, IF, and methotrexate, Turci et al. (21) reported an LOD of 0.05 ng/mL for CP and 0.2 ng/mL for IF; therefore, the current work has comparable LOD and the capability of quantitating 4-keto-CP. The LOD for 4-keto-CP was an improvement from that reported by Kasiel et al. (14); they reported an LOD of 5 ng/mL for their HPLC–MS procedure. Therefore, the current work should represent a reasonable method to support occupational safety and health studies of healthcare workers exposed to CP or IF. Also, a deuterated analog of CP was chosen for use as an internal standard for this
method. It would not be present in a worker exposed to the target drugs or their metabolites, and its use compensates for any individual differences in extraction variation or matrix ion suppression response with the MS detector.

**Extraction conditions development**

Ethyl acetate extraction was ultimately chosen for this liquid–liquid extraction (LLE) procedure owing to its superior extraction efficiency for 4-keto-CP, and its good extraction efficiency of CP and IF. Recovery was found to be 84% (n = 6) for 4-keto-CP, 85% (n = 6) for CP, and 86% (n = 6) for IF. Various solid-phase extraction procedures were tried during the early stages of this procedure, including the Biotage Isolute ENV+ (Charlottesville, VA), the Phenomenex Strata C18 (Torrance, CA), and the Varian Bond Elut C18 (Harbor City, CA). All performed nearly as well for the extraction efficiency of CP and IF, but the extraction efficiency was lower for 4-keto-CP (67–69%) for the solid-phase extraction cartridges tested. Ultimately, the lower cost of LLE over the specialized solid-phase extraction cartridge types, and the higher extraction efficiency of LLE for 4-keto-CP was chosen for this procedure. Also, it was necessary to buffer urine samples to a roughly neutral pH to obtain the best recovery of the target analytes. Acidic conditions gave lower extraction of CP and IF. The addition of 0.5 mL of 2 M potassium phosphate buffer (pH 7) to 4 mL of urine was found to give the high extraction efficiencies reported.

Other metabolites of IF were also evaluated for incorporation into this procedure. It was hoped that 4-ketoifosfamide (4-keto-IF, Niomech) could also be studied for possible use as a biomarker for IF exposure. Unfortunately, 4-keto-IF was found not to be stable under the aqueous buffer conditions of this procedure (pH 7) during this study. It was not stable in human urine under physiological conditions and, therefore, was a poor metabolic candidate for use as a biomarker of exposure. 2,3-Dechloroethylifosfamide (Niomech) was also initially evaluated during the early stages of method development. 2,3-Dechloroethylifosfamide extracted well with solid-phase extraction but poorly with various LLE conditions, and its chromatographic recovery was poor because of the lack of an internal standard specific for that compound to compensate for ion suppression. Due to the lack of an available deuterated standard, this metabolite was dropped from consideration for further study.

**Precision and accuracy of the optimized conditions**

A primary recovery experiment using the optimized procedure and blank urine fortified with 4-keto-CP, CP, and IF was performed over three separate analytical batch runs to demonstrate the accuracy and precision of the procedure. These data are presented in Table I. Average recovery ranged from 97 to 105% for the three levels of 4-keto-CP investigated, from 101 to 105% for the three levels of CP, and from 100 to 102% for the three levels of IF. For each analytical run, the experimental trial consisted of three urine samples prepared at three concentration levels. The recovery results for all three analytes are within the statistical expectation and do not appear to have any significant bias. This is consistent with what has been recently reported by Sottani et al. (9); CP and IF recovery ranged from 100 to 106% in a concentration range of 1 to 3.5 µg/mL in an HPLC–MS–MS method they developed. The greatest relative standard deviation (RSD) for the current work was 7.9% for the 1 ng/mL level of IF. Most of the variation in recovery can be attributed to the extraction step within the sample preparation step of this test procedure. The secondary recovery experiment used urine samples from 20 non-exposed volunteers, and it again demonstrated the procedure to be both accurate and precise (Table II). The 50 ng/mL fortified 4-keto-CP samples had a recovery of 105%. The 2 ng/mL CP and IF fortified samples showed a recovery of 104 and 103%, respectively. RSDs were 8.4% for 4-keto-CP, 2.6% for CP, and 7.2% for IF for the second recovery experiment. Both recovery experiments generally displayed less precision for 4-keto-CP and IF probably because of the use of only one deuterated analog of CP as the internal standard. The RSD of 4-keto-CP and IF were still within an acceptable range for use as a biomarker assay.

### Table I. Multiple Level Recovery Experiment of 4-Keto-cyclophosphamide, Cyclophosphamide, and Ifosfamide*

<table>
<thead>
<tr>
<th>Analyte/nominal conc. (ng/mL)</th>
<th>Mean measured conc. (n = 9)</th>
<th>Average % recovery (accuracy)</th>
<th>Standard deviation (ng/mL)</th>
<th>%RSD†</th>
</tr>
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<tbody>
<tr>
<td><strong>4-Ketocyclophosphamide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>24.2</td>
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<td>100</td>
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<td>375</td>
<td>386</td>
<td>103</td>
<td>13</td>
<td>3.4</td>
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<td><strong>Cyclophosphamide</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.05</td>
<td>105</td>
<td>0.056</td>
<td>5.3</td>
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<td>15</td>
<td>15.2</td>
<td>101</td>
<td>0.35</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Ifosfamide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>100</td>
<td>0.079</td>
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</tr>
<tr>
<td>4</td>
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<tr>
<td>15</td>
<td>15.2</td>
<td>101</td>
<td>0.37</td>
<td>2.4</td>
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</table>

* Three different spiked samples were prepared at each level and chromatographed on three separate experimental trial runs (a total of nine samples at each spike level were analyzed). The same Zorbax RX C18 column was used for experimental batch trials 1 and 2; a second Zorbax RX C18 column was used on trial run 3.

† %RSD = percent relative standard deviation.

### Table II. Recovery of 4-Ketocyclophosphamide, Cyclophosphamide, and Ifosfamide Spikes from Urine Samples of 20 Non-exposed Volunteers*

<table>
<thead>
<tr>
<th>Analyte mean measured conc. (ng/mL)</th>
<th>Average percent recovery (accuracy)</th>
<th>Measured conc. range (low to high) (ng/mL)</th>
<th>%RSD†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4-Ketocyclophosphamide</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>53.7</td>
<td>105</td>
<td>44.3–60.3</td>
<td>8.4</td>
</tr>
<tr>
<td><strong>Cyclophosphamide</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2.08</td>
<td>104</td>
<td>1.97–2.14</td>
<td>2.6</td>
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<tr>
<td><strong>Ifosfamide</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2.14</td>
<td>103</td>
<td>1.80–2.59</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* 4-Ketocyclophosphamide prepared theoretical conc. was 51.0 ng/mL, cyclophosphamide theory was 2.00 ng/mL, and cyclophosphamide theory was 2.08 ng/mL.

† %RSD = percent relative standard deviation.
Method reproducibility, analyte stability, and other considerations
Two different Agilent Zorbax RX C18 columns were used during the recovery studies. These results are, therefore, expected to be consistent and reproducible with HPLC columns from this manufacturer. This would indicate robustness for the chromatographic conditions used in this procedure. A five-day stability study was conducted on the final chromatographic sample solution. 4-Keto-CP, CP, and IF appeared to be stable at 7°C (the autosampler temperature) and at room temperature in open laboratory light. The procedure was found to be linear within the standard concentration ranges described: 10–625 ng/mL 4-keto-CP equivalent levels and 0.5–25 ng/mL CP and IF equivalent levels in urine. Correlation coefficients were 0.99 or greater, and y-intercepts approached zero for all curves generated with this procedure.
Planned future work includes the analysis of actual urinary samples collected from healthcare workers who may be exposed to CP or IF. This field sample work is part of a larger comprehensive study by this laboratory, which is beyond the scope of this work. It will be reported in detail elsewhere. This communication describes in detail the development, design, and validation of the assay procedure.

Conclusions
An accurate, precise, and reproducible HPLC–MS–MS assay procedure has been developed and validated for the simultaneous quantification of 4-keto-CP, CP, and IF. LLE of urine using ethyl acetate proved to be simple and gave high extraction efficiencies for all three analytes. The accuracy of this procedure was found to be good. Recoveries of 97–105% of theory were obtained for the target analytes with RSDs no higher than 8.4%. Standard curve data generated linear responses (correlation coefficients of 0.99 or greater) in the range 10–625 ng/mL for 4-keto-CP and in the range 0.5–25 ng/mL for CP and IF. The LOD was determined to be 1 ng/mL for 4-keto-CP, 0.1 ng/mL for CP, and 0.05 ng/mL for IF.

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