BACKUP DATA REPORT
NIOSH Method No. 8327

Title: Cyclophosphamide, 4-ketocyclophosphamide and ifosfamide in urine

Analyte: Cyclophosphamide, 4-ketocyclophosphamide and ifosfamide

Author/developer: Clayton B'Hymer

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Clayton B'Hymer

Substance(s):
1. Cyclophosphamide
2. 4-Ketocyclophosphamide
3. Ifosfamide

Exposure Limits: Not applicable

Chemical Used for Evaluation:
1. Cyclophosphamide monohydrate from Sigma Chemical, Inc., assay 97 to 103%, Lot no. 113K1406.
2. 4-Ketocyclophosphamide from Niomech IIT, GmbH, 98%, Lot no. D-18845.
3. Ifosfamide from American Pharmaceutical Partners (APP), Inc., 98%, Lot no. 200969.

GENERAL

Synopsis

Cyclophosphamide (CP) and ifosfamide (IF) are two commonly used drugs for the treatment of cancer. The two drugs are widely used by healthcare workers associated with cancer patients including nurses and pharmacy personnel who prepare dosages. The metabolism of these drugs is complex, each resulting in more than a dozen active and inactive metabolites [1, 2]. Some of these metabolites are unstable in urine [3] and therefore unsuitable for biomonitoring. The described procedure was developed for the detection and quantification of the two parent drugs and one of the inactive metabolites, 4-ketocyclophosphamide (4-keto-CP) in urine. It is hoped that this metabolite will serve as a good marker for exposure. Urine from an exposed population is collected, shipped cold, and stored frozen at -70 to -80 °C. The samples are thawed for analysis. Liquid-liquid extraction (LLE) using ethyl acetate collects the three analytes from the urine samples. The ethyl acetate extracts are evaporated and the dry residues are dissolved in water before high-performance liquid chromatographic (HPLC) analysis. Detection is performed by means of a tandem mass spectrometer (MS/MS). The deuterated analog of cyclophosphamide (d_6-CP) is used as an internal standard.

Method Evaluation

This method was evaluated in the general areas of a typical method validation [4, 5]. Furthermore, this method has been described by B'Hymer [6] and published by B'Hymer and Cheever [7]. The key elements of method validation including accuracy, precision, linearity, specificity, robustness, stability and limit of detection have been investigated during this method's development. The accuracy and precision were determined by spiked urine sample recovery studied at four different concentration levels and are described in detail later in this report. The other elements are also described in detail with their respective results within this report.

Sampling Aspects

All urine samples were prepared by mixing 4.0 mL of urine with 0.5 mL of 2 M potassium phosphate buffer solution (pH 7.0). The spiked urine samples were prepared by adding 0.25 mL of the appropriate spike-level solution to the buffered urine solution. Non-spiked samples, including the actual field samples, were diluted with 0.25 mL of water. All samples were spiked with a 0.25 mL aliquot of 48 ng/mL deuterated CP, which is the internal standard. Solids in urine samples caused no problems during the liquid-liquid extraction; therefore, filtering the urine was not included in this test method's sample preparation procedure.

Analytical Aspects

The chromatographic analysis was carried out using an Agilent Technologies model 1100 HPLC pumping system with autosampler (Palo Alto, California, USA) with an Agilent Technologies model 6410A triple quadrupole mass spectrometer used as the detector. The detector output was processed by Agilent's Mass Hunter software where all data were evaluated and the chromatographic peaks were integrated. The column used was an Agilent Zorbax Rx-C18 (150 X 3 mm, 3.5 µm particle size) using gradient elution with acetonitrile/water mobile phases containing 0.1% acetic acid. The chromatographic and mass spectrometric conditions were optimized for this method and are described below.

Chromatographic Conditions

Mobile Phases: A = 15/85/0.1% (v/v/v) acetonitrile/water/acetic acid
B = 75/25/0.1% (v/v/v) acetonitrile/water/acetic acid

Flow Rate: 0.3 mL/min

Gradient Program:

Time (min)	Mobile Phase Composition	Comments
0 to 2	0% B	Initial hold
2 to 10	0 to 30% B	First linear gradient step
10 to 18	30 to 100% B	Second linear gradient step
18 to 25	100% B	Final hold
25 to 27	100 to 0% B	Start re-equilibration
27 to 35	0% B	Column re-equilibration

Injection Volume: 10 µL

Mass Spectrometric Conditions

Ionization Source: Electrospray at 3000 Volts and positive scan mode, nebulizer gas at 35 psi and 10 L/min flow

Multiple Reaction Mode (MRM)**:**

Quantification mass transitions - CP = *m/z* 261 → 140, 4-keto-CP = 275 → 106, IF = 261 → 154, d_6-CP = 267 → 140.

Qualification mass transitions - CP = *m/z* 261 → 106, 4-keto-CP = 275 → 204, IF = 261 → 92,

The following table lists the instrumental settings used for the Agilent 6410A mass spectrometer:

Table 1: Mass spectrometer settings

Analyte	Precursor Ion	MS1 Resolution	Product Ion	MS2 Resolution	Dwell Time (msec)	Fragmentor Voltage	Collision Energy (volt)
d_6-CP	267	wide	140	unit	200	140	25
CP	261	wide	140	unit	200	140	25
CP	261	wide	106	unit	200	140	25
IF	261	wide	154	unit	200	140	25
IF	261	wide	92	unit	200	140	25
4-keto-CP	275	wide	106	unit	200	140	25
4-keto-CP	275	wide	204	unit	200	140	25

Extraction/Sample Preparation

Non-spiked urine samples and those spiked with the three analytes were treated identically. A 4.0 mL portion of the urine was placed in a screw-capped tube and buffered by the addition of 0.5 mL of 2 M potassium phosphate buffer (pH 7). A 0.25 mL aliquot of a 48 ng/mL d_6-CP internal standard solution was added. A 0.25 mL portion of deionized water or the appropriate analyte spiking solution was added. The urine sample was extracted three times with 5.0 mL of ethyl acetate using a vortex mixer for one minute for each extraction. The ethyl acetate layers were removed and combined. The extract solution was evaporated to dryness by means of a rotary concentrator at 30 °C. Prior to HPLC analysis, the dry extract was dissolved in 250 µL of deionized water and transferred to a 100 µL insert in an HPLC autosampler vial.

The chemical reagents used were those commonly found in a laboratory. The ethyl acetate was HPLC grade. The sources of the reference compounds have been described previously.

Method developmental considerations

Ethyl acetate was chosen for this method due to its improved extraction efficiency for 4-keto-CP as compared to solid phase extraction and its acceptable extraction efficiency for CP and IF. Recovery was found to be 84% (n=6) for 4-keto-CP, 85% for CP and 86% for IF using the method as described above. Other extraction procedures were evaluated during the early development stage of this method. Various solid-phase extraction procedures were evaluated and included Biotage Isolute ENV+,

Phenomenex Strata C18 and Varian Bond Elut C18 SPE cartridges. 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%). Also, it was necessary to buffer urine samples to roughly neutral pH to obtain the best recovery of the target analytes. Acidic conditions, which is usual for urine, 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.

Metabolites of IF were also evaluated for incorporation into this procedure. It was hoped that 4-ketoifosfamide (4-keto-IF) 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 was also initially evaluated during the early stages of method development. 2,3-Dechloroethylifosfamide extracted well with SPE, but poorly with various LLE conditions, and its chromatographic recovery was poor owing to the lack of an internal standard specific for that compound to compensate for ion suppression.

The only other minor difficulty found during the development of this method was with minor sample carry-over by the autosampler. The use of a needle rinse with 50/50 (v/v) acetonitrile/water was found to be necessary to eliminate this problem.

RESULTS

Accuracy and Precision

Two recovery studies using two columns over several days demonstrated the accuracy and precision of this test method. The recovery studies were performed using spiked urine samples containing known levels of CP, 4-keto-CP, and IF. The first recovery study was performed over three separate experimental batch runs, and these data are presented in Table 2. For each analytical run, the experimental trial consisted of three urine samples prepared at three concentration levels. 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. The recovery results for all three analytes are within the statistical expectation and do not appear to have any significant bias. The greatest percent relative standard deviation (%RSD) 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 process 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 3). The 50 ng/mL fortified 4-keto-CP samples had a recovery of 105%. The 2 ng/mL CP and IF fortified samples showed recoveries 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 owing to the use of only the one deuterated analog of CP as the internal standard. The %RSD of 4-keto-CP and IF were within an acceptable range for use as a biomarker assay.

Table 2

Multiple Level Recovery Experiment of 4-Keto-CP, CP and IF

Analyte/Nominal conc. (ng/mL)	Mean measured conc. (n=9) (ng/mL)	Average recovery (%)	Standard deviation (ng/mL)	%RSD[A]
4-Keto-CP				
25	24.2	97	1.2	5.1
100	105	105	3.4	3.2
375	386	103	13	3.4
CP				
1	1.05	105	0.056	5.3
4	4.10	103	0.094	2.3
15	15.2	101	0.35	2.3
IF				
1	1.00	100	0.079	7.9
4	4.08	102	0.085	2.1
15	15.2	101	0.37	2.4

Notes: 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.

[A] %RSD: percent relative standard deviation

Table 3

Recovery of 4-Keto-CP, CP and IF Spikes from Urine Samples of 20 Non-exposed Volunteers

Analyte mean measured conc. (ng/mL)	Average recovery (%)	Measured conc. range (low to high) (ng/mL)	%RSD[A]
4-Keto-CP			
53.7	105	44.3 – 60.3	8.4
CP			
2.08	104	1.97 – 2.14	2.6
IF			
2.14	103	1.80 – 2.59	7.2

Notes: 4-Keto-CP prepared theoretical concentration was 51.0 ng/mL, CP theory was 2.00 ng/mL and IF theory was 2.08 ng/mL

[A] %RSD: percent relative standard deviation

Linearity

All calibration curves used during the development of this method were found to be linear and had coefficients of determination of 0.99 and greater with y-intercepts close to zero. The standard concentration ranges described were 10 to 625 ng/mL 4-keto-CP equivalent levels and 0.5 to 25 ng/mL CP and IF equivalent levels in urine.

Specificity

The optimized chromatographic conditions developed for this procedure proved to be specific, have no major interferences and enabled 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 d_6-CP internal standard at the selected mass transitions used for quantification. 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 minutes into the gradient program. Since CP eluted at approximately 14 minutes, a reasonable baseline was obtained during its retention time window. Specificity and lack of interferences would be expected because of the use of the triple quadrupole mass spectrometer for detection.

While qualifying ions were not included in the final method, they were investigated during the development of the method. The qualifying mass transition for CP and IF were selected as the second highest response that did not have significant interferences with the existing components typically found in urine. The qualifying mass transition for 4-keto-CP was chosen for its unique signal for the compound. The mass transitions for qualification of the analytes can only be used at higher concentration levels, since they have a lower response than the quantifying mass transition ions.

Robustness

Two Agilent Zorbax Rx-C18 columns were used during the recovery studies. Accuracy and precision did not appear to be affected; therefore, the method appears to be reproducible with any functioning Zorbax Rx-C18 HPLC column. Recovery results from individual urine samples spiked with the three analytes indicate that the method was accurate and not significantly affected by individual urine sample matrix differences during analyte extraction or chromatographic analysis.

Stability

Sample stability was not exhaustively evaluated. Stock standard solutions of CP, 4-keto-CP and IF were made in acetonitrile and appeared to be stable after weeks of storage under refrigeration. A five-day stability study was conducted on the final chromatographic sample solution. The three analytes were stable at 7 °C (the autosampler temperature) and at room temperature in typical laboratory light (Table 4). Experiments conducted by Kasel, et al. [8] showed that CP and 4-keto-CP in urine were stable following three freeze-and-thaw cycles, were stable at room temperature for the time it takes to process the samples (six hours), and that the processed samples were stable after storage in the autosampler tray at 5 °C for 18 hours. It is reasonable to assume that ifosfamide would behave similarly. Other data also show the stability of CP and IF in urine [9,10].

Table 4

Stability of the analytes stored five days at two temperatures

Analyte	Recovery stored at 7 °C (%)	Recovery stored at room temperature (%)
CP	99.0	97.0
IF	99.1	99.5
4-keto-CP	104.8	100.1

Range

The procedure was found to be linear within the standard concentration ranges described; 10 to 625 ng/mL 4-keto-CP equivalent levels and 0.5 to 25 ng/mL CP and IF equivalent levels in urine. Field samples at higher levels can be diluted to a concentration within that range for analysis.

Limit of detection

The limit of detection (LOD) was calculated in a traditional manner [1,2] using three times the noise level divided by the slope of the calibration curve. Since 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 the 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 height was used only for the estimation of the LOD; the peak area ratio was used for quantification of the analytes during the validation of this procedure. This “instrumental” LOD was found to be approximately 1 ng/mL for 4-keto-CP, 0.1 ng/mL for CP and 0.05 ng/mL for IF. The actual LOD is dependent upon the performance of the chromatographic system and the detector at the time of the analysis and the extraction efficiency. Since this method calls for the lowest standard concentrations of 0.5 ng/mL for CP and IF and 10 ng/mL for 4-keto-CP, these can be considered the “operational” LODs and a basic criteria for the use of this method. If a column, chromatographic system or detector cannot detect the lowest standard level, corrective action would be required.

Ruggedness

Laboratory-to-laboratory reproducibility was evaluated through the User Check process. A laboratory was contracted to set up the method in their laboratory, show analytical proficiency, and then analyze a set of blind spiked samples. The results of this User Check and discussion of it are found in the Appendix.

References

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Appendix

Review of User Check for NMAM Method 8327 (Cyclophosphamide, 4-ketocyclophosphamide, and ifosfamide in urine)

User check samples were prepared by a BHAB researcher to be analyzed by a contract lab using draft NMAM Method 8327. A total of 25 urine samples were prepared. The urine was obtained from personnel in the Taft building at NIOSH and then combined and mixed in the BHAB labs into a single pool of urine from which all samples were prepared. Five samples were left blank. Five samples were prepared containing the analytes at each of the following levels: cyclophosphamide (CP) – 0.988 ng/mL, 1.98 ng/mL, 9.88 ng/mL, and 19.8 ng/mL; 4-ketocyclophosphamide (4-ketoCP) – 26.1 ng/mL, 52.2 ng/mL, 261 ng/mL, and 522 ng/mL; ifosfamide (IF) – 1.02 ng/mL, 2.04 ng/mL, 10.2 ng/mL, and 20.4 ng/mL. Thus the lowest level sample contained 0.988 ng/mL CP, 26.1 ng/mL 4-ketoCP, and 1.02 ng/mL IF and so forth for the other three concentration levels. The samples were prepared, shipped frozen on July 26, 2017, and arrived at the contract lab the next day. The samples were analyzed on August 7, 2017. The results are in Tables 1a-3a.

For this analysis, the Lower Limit of Quantitation (LLOQ) was determined by the lab to be ~0.5 ng/mL for CP and IF and ~10 ng/mL for 4-ketoCP. As mentioned above, the spike levels ranged from 1 to 20 ng/mL for CP and IF and from 25 to 500 ng/mL for 4-ketoCP which are 2 to 40 times the LLOQ and fall within the method detection range for all three analytes. The urine blanks showed no levels of any of the compounds, thus no adjustment was needed.

Summary tables for each analyte are shown below. Larger tables containing each individual sample value can be found at the end of this report as Tables 4a-6a.

Table 1a. Summary Cyclophosphamide user check results

Spiked amount (ng/mL)	Average recovery (%)	RSD (%)
19.8	111	2.6
9.88	114	1.9
1.98	120	2.8
0.988	120	3.3
Overall	116	4.3

Table 2a. Summary 4-Ketocyclophosphamide user check results

Spiked amount (ng/mL)	Average recovery (%)	RSD (%)
522	100	4.1
261	111	3.5
52.2	119	1.9
26.1	114	4.3
Overall	111	7.1

Table 3a. Summary Ifosfamide user check results

Spiked amount (ng/mL)	Average recovery (%)	RSD (%)
20.4	102	3.3
10.2	104	1.7
2.04	111	3.9
1.02	112	4.2
Overall	107	5.4

Two of the primary guidelines on bioanalytical method validation recommend that accuracy and precision should be within ± 15% at each level and within ± 20% at the lowest level [1,2]. The precision, as measured by relative standard deviation, fall within this range for every level of every analyte as well as for the overall precision across every sample for all three analytes. As a further acceptability proof of the precision of the method, the contract lab found that the inter-run RSD for the urine standards were 10% or less at all concentrations, that the intra-run RSDs for the urine quality control (QC) samples were 10% or less for all three compounds, and that the inter-run RSDs for the urine QC samples were within 10% for all three compounds. These inter-run values are produced over three separate analysis batches with four replicates of QC samples at three concentrations and performed by two different analysts.

The recovery values (accuracy) are slightly more mixed. The same three separate analysis batches mentioned above produced the following data. The intra-run relative errors (REs) for the urine standards were 10 percent or less at all concentrations for all compounds except the LLOQ which was up to -11.5 percent for IF and -10.6 percent for 4-keto-CP. The inter-run REs for the urine standards for all runs were 10 percent or less for all concentrations for all compounds. The intra-run REs for the urine QCs for all runs were 12 percent or less for all concentrations for CP and IF. The intra-run REs for the urine QCs for all 4-Keto-CP runs were 15.8 percent or less for all concentrations. The inter-run REs for the urine QCs were within 10 percent for all compounds.

For the blind User Check samples, the average recovery for ifosfamide is also within the desired range at every level and for the average of every sample, with a range of recovery from 96% to 120%. The

accuracy values for CP are slightly outside the recommended range at the next to lowest level and right at the top of the range at the lowest level with recoveries ranging from 106% to 125%. The values for 4-keto-CP are also slightly above the recommended range at the next to lowest level with recoveries ranging from 97% to 122%. While the high recoveries at a couple of individual levels are somewhat less than desirable for this User Check, the multitude of the data in this User Check, in the method development, and in the contract lab's multi-day runs demonstrate an acceptable level of precision and accuracy.

The contract lab reported no difficulties understanding the draft method nor in setting it up or analyzing the samples. The method has relatively few analytical steps, is quite straightforward, is sensitive enough to determine occupational exposures to CP and IF, and has been shown to have adequate precision and accuracy. It is recommended that the method, NMAM Method 8327 (Cyclophosphamide, 4-ketocyclophosphamide, and ifosfamide in urine) be approved and accepted for inclusion in the NIOSH Manual of Analytical Methods.

Dale Shoemaker, PhD
Research Chemist
August 10, 2018

References

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Table 4a. Cyclophosphamide user check results

CP found (ng/mL)	Target (ng/mL)	Recovery (%)
22.6	19.8	114.1
22.1	19.8	111.6
21.9	19.8	110.6
22.2	19.8	112.1
20.9	19.8	105.6
10.9	9.88	110.3
11.5	9.88	116.4
11.3	9.88	114.4
11.5	9.88	116.4
11.3	9.88	114.4
2.38	1.98	120.2
2.43	1.98	122.7
2.44	1.98	123.2
2.34	1.98	118.2
2.26	1.98	114.1
1.19	0.988	120.4
1.24	0.988	125.5
1.20	0.988	121.5
1.19	0.988	120.4
1.12	0.988	113.4

Table 5a. 4-Ketocyclophosphamide user check results

4-ketoCP found (ng/mL)	Target (ng/mL)	Recovery (%)
565	522	108.2
516	522	98.9
517	522	99.0
516	522	98.9
504	522	96.6
281	261	107.7
303	261	116.1
281	261	107.7
279	261	106.9
298	261	114.2
62.8	52.2	120.3
62.2	52.2	119.2
63.2	52.2	121.1
59.8	52.2	114.6
62.3	52.2	119.3
29.0	26.1	111.1
30.5	26.1	116.9
28.2	26.1	108.0
29.6	26.1	113.4
31.9	26.1	122.2

Table 6a. Ifosfamide user check results

IF found (ng/mL)	Target (ng/mL)	Recovery (%)
21.5	20.4	105.4
21.3	20.4	104.4
20.9	20.4	102.5
20.4	20.4	100.0
19.6	20.4	96.1
10.4	10.2	102.0
10.8	10.2	105.9
10.4	10.2	102.0
10.7	10.2	104.9
10.8	10.2	105.9
2.24	2.04	109.8
2.36	2.04	115.7
2.38	2.04	116.7
2.19	2.04	107.4
2.16	2.04	105.9
1.08	1.02	105.9
1.22	1.02	119.6
1.15	1.02	112.7
1.16	1.02	113.7
1.11	1.02	108.8