Formula: 1. Cyclophosphamide, $C_7H_{15}Cl_2N_2O_2P$	MW: 1. 261.1	CAS: 1. 50-18-0	RTECS: 1. RP5950000
2. 4-Ketocyclophosphamide, $C_7H_{13}Cl_2N_2O_3P$	2. 275.1	2. 27046-19-1	2. none
3. Ifosfamide, $C_7H_{15}Cl_2N_2O_2P$	3. 261.1	3. 3778-73-2	3. RP6050000

METHOD: 8327, Issue 1 **EVALUATION:** FULL **Issue 1:** 5 March 2021

OSHA: None
NIOSH REL: None
Because data on exposure limits and guidelines may change over time, NIOSH recommends referring to the following sources for updated limits and guidelines [1-3].

PROPERTIES: 1. white, crystalline powder; MP 41-45 °C or 47-49 °C or 49.5-53 °C [4,5] (Cyclophosphamide is often purchased as the monohydrate, CAS 6055-19-2. These various melting points reflect both the compounds.) 2. white solid; MP 139-141 °C [6] 3. white crystalline powder, MP 39-41 °C [4,5]

SYNONYMS: 1. *N*,*N*-bis(2-chloroethyl)-2H-1,3,2-oxazaphosphorin-2-amine-2-oxide or 2-(bis(2-chloroethyl)amino)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide.; 2. 4-oxycyclophosphamide or 2-(bis(2-chloroethyl)amino)tetrahydro-4H-1,3,2-oxazaphosphorin-4-one 2-oxide.; 3. N,3-bis-(2-chloroethylamino)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide or (2-chloroethylamino)-N-(2 chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide.

SAMPLING

SPECIMEN: Urine

VOLUME: At least 8 mL of sample

PRESERVATIVE: None

SHIPMENT: Frozen

SAMPLE STABILITY: Not established. Appears to be stable in Frozen urine for long periods. See [7] for more details.

CONTROLS: Urine samples obtained from non-exposed individuals

ACCURACY [7]

RANGE STUDIED: 1 to 20 ng/mL for CP and IF, 25 to 525 ng/mL for 4-keto-CP

BIAS: None established

OVERALL PRECISION ($\hat{S}_{rT}$): See Tables 1 and 2

ACCURACY: 103% recovery for cyclophosphamide and 4-ketocyclophosphamide, 102% recovery for ifosfamide. The precision by relative standard deviation was no larger than 8.4% at any concentration level (see Tables 1 and 2).

MEASUREMENT

TECHNIQUE: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY (HPLC/MS/MS)

ANALYTES: Cyclophosphamide (CP), 4-ketocyclophosphamide (4-keto-CP) and ifosfamide (IF)

EXTRACTION: Liquid-liquid extraction with ethyl acetate

COLUMN: C18 (150 X 3 mm, 3.5 µm).

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:

Time (min)	Mobile Phase Composition
0 to 2	0%B
2 to 10	0 to 30% B
10 to 18	30 to 100% B
18 to 25	100% B
25 to 27	100 to 0% B
27 to 35	0% B

INJECTION VOLUME: 10 µL

IONIZATION SOURCE: Electrospray at 3000 Volts and positive scan mode, nebulizer gas at 35 psi and 10 L/min.

DETECTOR (MS/MS): Dwell time = 200 msec
Fragmentor at 140 Volts
Collision energy at 25 Volts.
Nitrogen collision gas.

CALIBRATION: Standards from CP, 4-keto-CP and IF spiked urine solutions with internal standard.

RANGE: 0.5 to 25 ng/mL CP and IF; 10 to 625 ng/mL 4-keto-CP

ESTIMATED LOD: By IUPAC definition [8]: CP and IF = approximately 0.1 ng/mL, 4-keto-CP = approximately 1 ng/mL. LOD is dependent upon column and instrumentation. By lowest standard levels: CP and IF = 0.5 ng/mL, 4-keto-CP = 10 ng/mL.

PRECISION ($\bar{S}_r$): See Tables 1 and 2.

APPLICABILITY: CP and IF are drugs used to treat cancer; 4-keto-CP is a metabolite and biomarker for CP exposure. This method measures the quantity of the three target analytes in urine.

INTERFERENCES: None found or identified.

OTHER METHODS: This method is based upon that described by B'Hymer and Cheever [9]. A review article of LC/MS methods for urinary analysis of anticancer drugs may be found to be useful [10].

REAGENTS:

1. Cyclophosphamide (CP) monohydrate reference standard.* Prepare a 0.20 mg/mL stock solution in acetonitrile. Store in a refrigerator.
2. Ifosfamide (IF) reference standard.* Prepare a 0.20 mg/mL stock solution in acetonitrile. Store in a refrigerator.
3. 4-Ketocyclophosphamide (4-keto-CP) reference standard.* Prepare a 0.10 mg/mL stock solution in acetonitrile. Store in a refrigerator.
4. Deuterated cyclophosphamide-d_6 (d_6-CP) reference standard.* Prepare a 0.15 mg/mL stock solution in acetonitrile. Store in a refrigerator.
5. Internal standard spiking solution, d_6-CP stock solution diluted to approximately 48 ng/mL in water.
6. Acetonitrile, HPLC grade or better.*
7. Acetic Acid, glacial, LC/MS grade or better.*
8. Ethyl Acetate, HPLC grade or better.*
9. Acetonitrile/water (50/50, v/v) injector rinse solution.
10. Mobile phase A (15/85/0.1, v/v/v) acetonitrile/water/acetic acid.
11. Mobile phase B (75/25/0.1, v/v/v) acetonitrile/water/acetic acid.
12. Potassium hydroxide solution, 5 M. Purchase or prepare by dissolving 28.05 g KOH in 100 mL water.
13. Potassium phosphate buffer, 2 M (pH 7.0). Prepare by diluting 23.0 g H_3PO_4 (85%) to 100 mL with water. pH to 7.0 using 5 M KOH solution.
14. Potassium phosphate buffer, 0.02 M (pH 7.0). Prepare by diluting 2 M solution 1:100.
15. Water, doubly deionized.
16. Water, HPLC grade or better for use as mobile phase.

*See SPECIAL PRECAUTIONS.

EQUIPMENT:

1. High-Performance Liquid Chromatograph equipped with a tandem mass spectrometric detector
2. C18 HPLC column (150 X 3mm, 3.5 µm particle size)
3. Autosampler capable of maintaining 7 °C
4. Analytical balance
5. Rotary vacuum concentrator with cold trap and vacuum pump or nitrogen evaporator
6. Automatic pipettor with disposable tips in the 1000 µL volume delivery range
7. Test tube vortex mixer
8. Re-pipettor to dispense 5 mL volumes of liquid
9. Disposable screw-top culture tubes (16 X 150 mm) with PFTE-lined caps
10. Metal spatula
11. Volumetric flasks; 10, 50, 100 and 200 mL, glass
12. Autosampler vials, amber with caps, septa and 100 µL deactivated glass inserts
13. Disposable glass Pasteur pipets
14. Sintered glass filtering apparatus and 1-L side-armed Erlenmeyer flask for filtration of mobile phases
15. Filters, glass microfiber, size to fit filtering apparatus, 0.7 µm
16. pH meter

SPECIAL PRECAUTIONS: Wear appropriate gloves (nitrile, polyurethane, neoprene, or latex), lab coat, and safety glasses while handling all chemicals and human urine products. Standard precautions [11] should always be used when handling bodily fluids and/or extracts of bodily fluids.
Acetic acid, acetonitrile, and ethyl acetate are flammable; handle with care and use in a chemical fume hood. CP and IF are known carcinogens. 4-keto-CP, and d_6-CP are unable to be classified, but observe all the required safety precautions. Reagents with manufacturer expiration dates should be observed.

SAMPLING:

1. Collect the urine in a polypropylene tube or bottle and cap.
2. Ship the sample stored in either wet or dry ice in an insulated container, store frozen upon arrival at the laboratory

SAMPLE PREPARATION:

3. Thaw the urine sample to room temperature.
4. Mix thoroughly to ensure urine homogeneity.
5. Transfer 4.0 mL of urine into a 16 X 150 mm screw-capped culture tube.
6. Add 0.5 mL of 2 M potassium phosphate buffer solution.
7. Add 0.25 mL of the 48 ng/mL internal standard spiking solution.
8. Add 0.25 mL of deionized water.
9. Ethyl Acetate Extraction. Dispense 5 mL of ethyl acetate into culture tube, place cap on culture tube, and vortex for 1 minute. Inversion of the tubes may be substituted if vortexing causes too much emulsion to form (>1/2 inch or if it starts to affect the results).
10. Allow layers to separate, collect the ethyl acetate layer. If emulsions form, centrifugation of the samples may be performed to aid the layers in separating.
11. Repeat the extraction (steps 9-10) two more times. Collect and combine all ethyl acetate extracts using a Pasteur pipet.
12. Evaporate the combined ethyl acetate extract for each sample to dryness using a rotary concentrator or nitrogen evaporator at 30°C temperature.
    NOTE: If storing the dry extract before analysis, it is advised to store cold, in either a refrigerator or freezer (≤4°C).
13. Dissolve the extract in 0.25 mL of HPLC grade water. Transfer the sample into the HPLC autosampler vial containing the 100 µL insert and analyze per steps 24-28.

CALIBRATION AND QUALITY CONTROL

14. Prepare a CP/IF standard mixture solution by combining 1.0 mL of each stock solution and diluting to 50 mL with 0.02 M phosphate buffer to make a 4 µg/mL concentration for each analyte.
15. Prepare CP/IF/4-keto-CP spiking solutions. The 4 µg/mL CP/IF standard mixture (from Step 14) and the 0.1 mg/mL 4-keto-CP stock solution are diluted in 0.02 M phosphate buffer to make 8, 16, 32, 160, 320 and 400 ng/mL CP/IF and 0.2, 0.4, 0.8, 4, 8 and 10 µg/mL 4-keto-CP solutions for spiking. Prepare one additional 160 ng/mL 4-keto-CP solution for the low level standard spike.
16. Transfer 4.0 mL of non-exposed urine into a 16 X 150 mm screw-capped culture tube.
17. Add 0.5 mL of 2 M potassium phosphate buffer solution.
18. Add 0.25 mL of the 48 ng/mL internal standard (IS) solution.
19. Add 0.25 mL of the spiking solution described in step 15 to make urine samples equivalent to 0.5, 1, 2, 10, 20, 25, ng/mL of CP/IF and 10, 12.5, 25, 50, 250, 500 and 625 ng/mL of 4-keto-CP in the original 4.0 mL volume of urine. Blank urine that has none of the analytes is used for these standards (see step 21).
20. Process the spiked standard urine samples the same as described in the preceding Sample Preparation section using steps 9 through 13.

21. Prepare and process one blank urine without analyte spikes to verify the source of blank urine contained no detectable quantity of CP, IF, or 4-keto-CP.
22. Prepare and process at least one quality control (QC) standard of CP/IF/4-keto-CP fortified urine using separately weighed and prepared stock solutions. QC samples should be greater than 5% of the sample set. QC values should normally be within ±20% of the spiked values. If not, the batch is considered out of control, the batch data discarded, and corrective actions taken before more specimens are analyzed per steps 24-28.
23. Measure the peak areas of the analytes and the IS in the chromatograms. Divide the peak area of the analytes by the peak area of the deuterated IS (d_6-CP). Prepare calibration curves of this quotient versus the concentration of the standards for the three analytes. The standard curve should have a coefficient of determination (r^2) of equal to or greater than 0.98 to be acceptable for use. Furthermore, when each standard is inserted into the calibration equation, the value should be within ±20% of the expected value.

MEASUREMENT:

24. Set the high-performance liquid chromatograph according to the manufacturer's recommendations and to the conditions listed previously.
25. Set the mass spectrometric detector to MULTIPLE REACTION MODE (MRM). Quantification mass transitions are as follows: CP = m/z 261 → 140, 4-keto-CP = 275 → 106, IF = 261 → 154, d_6-CP = 267 → 140. Program MRM according to the manufacturer's recommendations. For an Agilent model 6410A, please see the additional conditions listed below:

MS/MS conditions using positive electrospray ionization

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
IF	261	wide	154	unit	200	140	25
4-keto-CP	275	wide	106	unit	200	140	25

26. Inject 10 µL of each sample extract, calibration standard, QC, and blank.
27. Rinse the injection needle with the 50/50 acetonitrile/water solution after every injection to prevent carryover.
28. Measure the peak areas of the three analytes (CP, IF and 4-keto-CP) and that for the IS in the chromatograms. Divide the peak area of the analytes by the peak area from the IS. A representative chromatogram of the three analytes of interest is shown below (Figure 1). The chromatogram does not show the IS, which elutes at nearly the same retention time (13.4 min) as the non-deuterated CP.

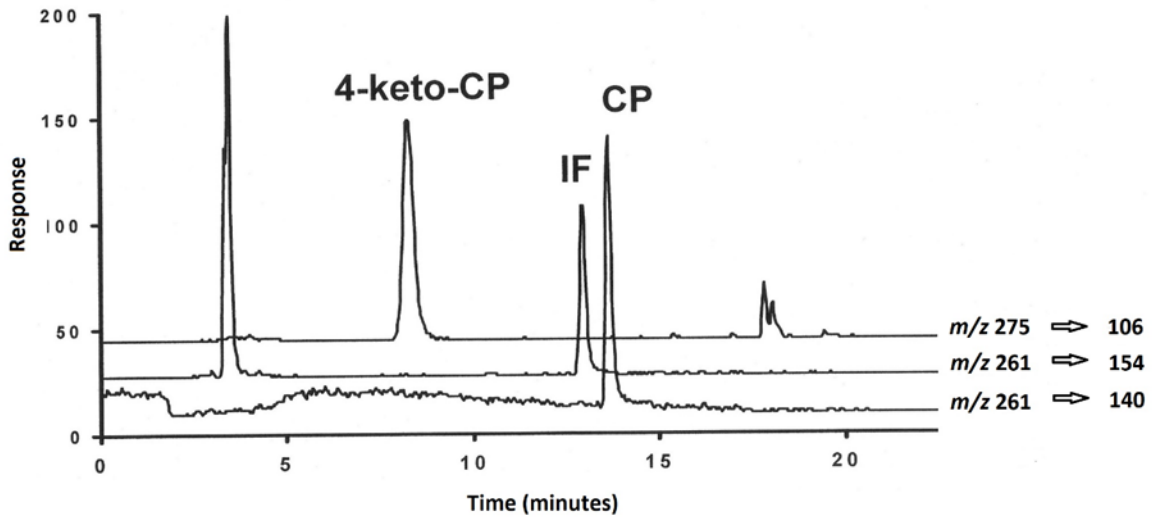

Figure 1. Chromatograms of urine fortified with 50 ng/mL 4-keto-CP (8.2 min), 2 ng/mL IF (12.9 min), and 2 ng/mL CP (13.6 min).

CALCULATIONS:

29. Determine the concentration of the three analytes in the extracts from the original urine (4.0 mL sample) from the curves obtained in step 23. The results can be expressed as ng/mL of each analyte in urine.

EVALUATION OF METHOD:

This method was evaluated and described in detail by B'Hymer and Cheever [9] and in the method backup data report [7]. A general summary of this previously published information is given below.

Accuracy and Precision. Two recovery studies using two columns from different lots performed over several days demonstrated the accuracy and precision of this test method. The two recovery studies generated a total of 47 spiked urine samples at 1, 2, 4, and 15 ng/mL CP/IF levels and 25, 50,100 and 375 ng/mL 4-keto-CP levels. The first recovery study used urine from volunteers that was pooled after collection and then fortified at the various levels. The study was performed over three separate experimental batch runs and these data are presented in Table 1. Average recoveries were between 97 and 105% for the three analytes over the concentration levels investigated. For each batch run, the experimental trial consisted of three samples at three different concentration levels. The recovery for each level (n=9 samples) is displayed in Table 1. The second recovery study used fortified urine samples from 20 non-exposed volunteers and demonstrated that the procedure was accurate and precise (Table 2). No interferences were detected in the unspiked urine from the 20 volunteer samples. Precision expressed as percent relative standard deviation (% RSD) was as high as 8.4% on the volunteer recovery samples (n=20). Overall recoveries had mean values of 102% (n=47) for IF and 103% (n=47) for both CP and 4-keto-CP; thus, there was no apparent bias.

Linearity. All calibration curves used during the development of this method were linear and had coefficients of determination of 0.99 and greater. The concentration range was 0.5 to 25 ng/mL CP and IF with a 4.0 mL urine sample size. The concentration range was 10 to 625 ng/mL 4-keto-CP in urine. Calibration curves were run at the beginning and end of all sample batch runs; calibration curve slope drift was found to be minimal during the development and validation of this method.

Specificity. The optimized chromatographic conditions developed for this method, along with the tandem mass spectrometric detector, proved to be specific and have no major interferences. The mass transition ions of CP and IF chosen in this method had the greatest response. This strategy gave the best sensitivity for those two analytes. The mass transition for 4-keto-CP, m/z 275 → 106, was chosen because it was more unique for the compound compared to the other possible metabolites of IF, which had similar chromatographic retention times. This method does not use qualifying ions as it is believed that retention times coupled with the multiple reaction mode offer enough specificity. Qualifying ions were investigated during the development of the method. Those transitions and some discussion can be found in the method backup data report [7].

Robustness. Two Agilent Rx-C18 HPLC columns of different manufacturing lots 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 Agilent Rx-C18 HPLC column. Recovery results from individual volunteer urine samples spiked with the three analytes indicate that the method is accurate and not significantly affected by individual urine sample matrix differences during analyte extraction.

Stability. Sample stability was evaluated. A five-day stability study was conducted on the final chromatographic sample solution. CP, IF, and 4-keto-CP appeared to be stable at 7 °C (the autosampler temperature) and at room temperature in laboratory light when stored in amber autosampler vials.

Range. This method should be considered accurate for the estimation of CP and IF in human urine within the 1 to 25 ng/mL range. It also should be considered accurate for the estimation of 4-keto-CP in human urine within the 25 to 525 ng/mL range. Field samples at higher levels can be diluted to a concentration within those ranges for analysis.

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METHOD WRITTEN (REVISED) BY:

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DISCLAIMER

TABLE 1

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

Analyte/Nominal conc. (ng/mL)	Mean measured conc. (n=9) (ng/mL)	Average percent recovery (Accuracy)	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 analyzed on three separate experimental trial runs (a total of nine samples at each spike level were analyzed). The same LC column was used for experimental batch trials 1 and 2; a second LC column was used on trial run 3.
[A] %RSD: percent relative standard deviation.

TABLE 2

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

Analyte mean measured conc. (ng/mL)	Average percent recovery (n=20) (Accuracy)	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: The prepared theoretical concentrations were 51.0 ng/mL for 4-Keto-CP, 2.00 ng/mL for CP, and 2.08 ng/mL for IF.
[A] %RSD: percent relative standard deviation.