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A Sensitive and Rapid Ultra High-Performance Liquid Chromatography with Tandem Mass Spectrometric Assay for the Simultaneous Quantitation of Cyclophosphamide and the 4-Hydroxycyclophosphamide Metabolite in Human Plasma

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Abstract

Analysis of cyclophosphamide (CP) and its metabolite, 4-hydroxycyclophosphamide (4OHCP), in a single assay has the ability to improve sampling techniques benefitting both the patients who are receiving the drug and the clinicians drawing samples. Due to instability in plasma ($t_{1/2} = 4$ minutes), immediate stabilization of 4OHCP with phenylhydrazine is necessary upon sample draw. After stabilization, 4OHCP and the stable CP prodrug concentrations can be analytically measured to elucidate the pharmacokinetics, including half-life and exposure parameters (C_{max} and AUC). For this purpose, a sensitive analytical method was developed to measure both the prodrug and active metabolite. A liquid-liquid extraction recovered the analytes prior to analysis with an ultra HPLC-MS/MS. A Thermo Scientific™ Hypersil™ BDS C18, 2.1×100 mm, 3.0 μm column was used for compound separation. Mass transitions for CP (m/z 261.0→140.0), the internal standard d4-CP (m/z 265.0→140.0), 4OHCP (m/z 367.3→147.1), and the internal standard AZD7451 (m/z 383.4→341.1) were monitored over a calibration range of 34.24–34,240 ng/mL and 3.424–3,424 ng/mL for CP and 4OHCP, respectively. Each calibration range proved accurate (<15% deviation) and precise (<15% RSD) for the desired compound. Using this method, CP and 4OHCP plasma levels can be measured in clinical samples from patients receiving this therapy.

Keywords

Cyclophosphamide; 4-hydroxycyclophosphamide; ultra-high performance liquid chromatography; tandem mass spectrometry; sickle cell disease

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1. Introduction

Cyclophosphamide (2-[Bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide) is a prodrug widely prescribed in the treatment of autoimmune disorders and various types of cancers[1, 2]. Hepatic oxidation of cyclophosphamide (CP) by multiple isozymes of cytochrome P450 (mainly CYP2B6 with CYP3A4, CYP2C, and CYP2A6 also contributing) [3] yields 4-hydroxycyclophosphamide (2-[Bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorin-4-ol 2-oxide)[1, 4]. Considered a transport metabolite, 4-hydroxycyclophosphamide (4OHCP) allows drug diffusion into target cells where further metabolism yields acrolein and the active metabolite, phosphoramidate mustard[4]. Therefore, systemic 4OHCP concentration serves as an excellent indicator of CP intracellular activation[2].

Due to very poor plasma stability ($t_{1/2}$ ~4 min), 4OHCP necessitates stabilization through a derivatization reaction[2]. Various agents have been employed for this reaction, including aryl hydrazines[3], semicarbazide[5], and hydroxylamines[6]. In this case, phenylhydrazine was the derivatizing agent of choice.

Prior validated assays have been published for measuring CP and 4OHCP, however, these methods were only capable of measuring a single analyte, were more cumbersome in their sample preparation procedure, or were less sensitive than the current method provided. Kalhorn et al. used LC-MS, but the lack of fragmenting created large background, thereby decreasing sensitivity and selectivity[4]. Using LC-MS/MS, Ekhardt et al. achieved greater selectivity, but the assay was relatively insensitive with an LOQ of 200 ng/mL for CP and 50 ng/mL for 4OHCP[5], much greater than the 34.24 and 3.424 ng/mL for each analyte, respectively, in this assay. For an enantioselective analysis of 4OHCP, de Castro et al. achieved an LOQ of 5 ng/mL, but CP was not measured, and enantiomeric separation necessitated a long run time of 20 minutes. Shu et al achieved a similar run time with simultaneous quantitation of both analytes with an LOQ of 5 ng/mL[7]. Several improvements in the extraction procedure and chromatographic techniques have been made compared to this reported assay. While the LOQ for our assay is only slightly lower than Shu et al demonstrate, far less plasma volume is needed to achieve it – 200 μ L versus 500 μ L. Furthermore, the extraction procedure has been streamlined from an ACN protein crash followed by an ethyl acetate liquid-liquid extraction to exclusively an MTBE extraction, thereby cutting preparatory time and the time samples potentially degrade. Finally, the reported assay uses the more sensitive uHPLC system rather than the older, less sensitive HPLC system. While the LOQ in both assays are similar – 5 ng/mL versus 3.424 ng/mL – the use of uHPLC gives much sharper peaks as exhibited by the LOD reaching under 1 ng/mL. Yang et al used a protein precipitation to analyze CP and one of its metabolites[8]. While the assay prep is rapid, it does not simultaneously measure CP and 4OHCP, and the assay only reaches an LOQ of 20 ng/mL. Another previous assay achieved an LOQ of 5 ng/mL, but again a much larger volume of plasma was necessary[9]. Other older published assays use HPLC-UV or gas chromatography, however these techniques are outdated and less sensitive.

In this report, a novel assay is presented that has the most sensitive 4OHCP LOQ (3.424 ng/mL) to date. Additionally, the rapid 3 min run time allowing for the simultaneous quantitation of both parent drug and active metabolite will allow pharmacokinetic studies to be performed from a single blood draw, which benefits patients. With increased sensitivity, clinicians will gain greater insight into the drugs' properties and how to effectively treat patients.

2. Materials and Methods

2.1. Materials

Cyclophosphamide was purchased from Sigma-Aldrich (St. Louis, MO). 4-hydroperoxy-cyclophosphamide, which is then reduced in water to the 4-hydroxycyclophosphamide metabolite, was purchased from Asta Medica (Frankfurt, Germany). Deuterated ($^2\text{H}_4$) cyclophosphamide (d4-CP) was purchased from Santa Cruz Biotechnology (Dallas, TX), while AZD7451 mesylate was a generous gift from AstraZeneca. All analyte and internal standard structures are presented in Figure 1.

Human whole blood (with EDTA) was provided by the National Institutes of Health Blood Bank. Optima-brand acetonitrile and methanol, and HPLC-grade methyl-tert-butyl ether (MTBE) were purchased from Fisher Scientific (Pittsburgh, PA). Phenylhydrazine, sodium citrate, sodium hydroxide, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). All water for the assay was deionized and ultra-filtered through a MilliPore system (EMD MilliPore, Billerica, MA).

2.2. Preparation of Stock Solutions

Phenylhydrazine was used as the derivatizing agent. A 4% w/v solution of phenylhydrazine was prepared in 0.1 M sodium citrate (aq) by dissolving 4 g of phenylhydrazine in 50 mL of the citrate buffer. The pH was adjusted to 6.0 using 0.5 M sodium hydroxide. Final volume was adjusted to 100 mL with water.

All master stock solutions are prepared such that the free (un-derivatized) drug concentration is 1 mg/mL. CP and d4-CP were prepared in acetonitrile. AZD7451 was prepared in methanol by adding 1.27 mg of AZD7451 mesylate for every mL of solvent. 4OHCP was prepared by adding 1.06 mg of 4-hydroperoxy-cyclophosphamide for every mL of water. Spontaneous reduction of 4-hydroperoxy-cyclophosphamide yields 4OHCP.

A 100 ug/mL working stock solution of phenylhydrazine-derivatized 4OHCP was prepared by adding 100 uL of the 1 mg/mL 4OHCP master stock into 900 uL of phenylhydrazine solution. After mixing, the solution is left at room temperature for 45 minutes to achieve maximum derivatization[2, 10].

Stock solutions were prepared from the 1 mg/mL master stock of CP, and the 100 ug/mL stock of derivatized 4OHCP. Both parent drug and metabolite were serially diluted from the same working stock to the following stock concentrations: 342.4, 684.8, 1,712, 8,560, 17,120, 34,240, 85,600, and 342,400 ng/mL for CP; and 34.24, 684.8, 171.2, 856, 1,720,

3,240, 8,560, and 34,240 ng/mL for derivatized 4OHCP. The 4% w/v phenylhydrazine solution was used as the solvent in the preparation of all standard stock solutions.

2.3. Sample Preparation

Initially, a solution of 4:3 whole blood to 4% phenylhydrazine solution was prepared. The solution was mixed through inversion, then centrifuged for 10 minutes at 2,000 RPM to remove hematocrit. The supernatant was removed and used as matrix for the preparation of calibration curves and quality control (QC) samples. Calibration standards were prepared by diluting the stocks 10-fold into matrix. Calibration ranges were 34.24–34,240 ng/mL and 3.424–3,424 ng/mL for CP and 4OHCP in plasma, respectively, after correction for diluting 3 parts blood into 4 parts phenylhydrazine solution. A hematocrit fraction in blood of 0.45 was assumed constant. Low, mid, and high QCs had a concentration of 102.72, 5,136, and 30,816 ng/mL and 10.272, 513.6, 3,081.6 ng/mL for CP and 4OHCP, respectively. A lower limit of quantitation (LLOQ) of 34.24 ng/mL and 3.424 ng/mL was included for the validation (n=20), as was a dilution QC (DQC) with concentrations of 85,600 ng/mL and 8,560 ng/mL for CP and 4OHCP, respectively. Because study sample concentrations exceeded the original DQC concentration, thereby making them unquantifiable, an additional DQC (the DHQC) was validated with concentrations of 684,800 ng/mL and 68,840 ng/mL for CP and 4OHCP, respectively.

To 200 μ L of sample supernatant, 1 mL of MTBE with IS (20 ng/mL of both internal standards) was added. Samples were vortexed for at least 1 min, then centrifuged for 10 minutes at 4°C and 13,300 RPM to separate the organic and aqueous layers. The organic supernatant was transferred to a 96 well 2 mL collection plate for drying. Samples were dried down under air for 15 minutes at 40°C prior to reconstitution in 45/55/0.2% v/v/v water/ACN/FA to match the initial concentration of the mobile phase. The plate was vortexed for 30 seconds to re-suspend analyte residue, followed by centrifugation for 10 minutes at 4°C and 2000 RPM.

2.4. Instrumentation and Chromatography

Ten microliters of each sample was injected onto a Waters ACQUITY UPLC® system (Waters Corporation, Milford, MA, USA), which included a binary pump, refrigerated autosampler (4 °C) and a temperature-controlled column compartment (35 °C). Chromatographic separation was achieved on a Thermo Scientific™ Hypersil™ BDS C18, 2.1×100 mm, 3.0 μ m column. Mobile phases were 0.2% formic acid (aq) and 0.2% formic acid in ACN. An isocratic mobile phase of 45/55/0.2 v/v/v H₂O/ACN/FA[2], with a flow rate of 0.35 mL/min was maintained for the entire 3 minute run. The column eluent was directed to an AB Sciex QTRAP 5500 mass spectrometer (AB Sciex, Framingham, MA). The diverter was initially set to waste for the first 0.5 minutes of the run. It was then switched to injecting into the mass spectrometer until 1.5 minutes when it was diverted back to waste for the duration of the run. The mass spectrometer monitored transitions of CP (m/z 261.0→140.0), the internal standard d4-CP (m/z 265.0→140.0), 4OHCP (m/z 367.3→147.1), and the internal standard AZD7451 (m/z 383.4→341.1) using multiple reaction monitoring (MRM) in the positive ion mode. The collision energy for each of the compounds was 18V, 35V, 13.26V, and 34V for CP, d4-CP, 4OHCP, and AZD7451,

respectively. Source parameters applicable to all analytes were a curtain gas of 30, collision gas (CAD) of medium, temperature of 550°C, and ion source gas 1/2 of 45. Multiquant™ Software version 2.0.2 was used for peak integration and data analysis (AB Sciex, Framingham, MA).

2.5. Validation

2.5.1. Linearity—Calibration curves for CP were constructed by least-squares quadratic regression analysis of an 8-point calibration range (34.24–34,240 ng/mL) by plotting the ratio of the analyte peak area versus the internal standard peak area, using $1/x^2$ as a weighting factor, where x is the ratio of the nominal analyte: IS concentration. Calibrator response functions and choice of regression analysis were investigated by calculating the correlation coefficient (r^2) and the percent deviation (% DEV, aka accuracy) for all calibrators.

Similarly, 4OHCP calibration curves used a linear regression analysis over an 8-point calibration range of (3.424–3,424 ng/mL). The same weighting factor and analysis as above were performed to determine the linearity of the 4OHCP calibration curves.

2.5.2. Accuracy and Precision—Accuracy and precision of both CP and 4OHCP were determined at 6 different quality control sample concentrations – lower limit of quantitation (LLOQ), low-range (LQC), midrange (MQC), high-range (HQC), 10-fold dilution (DQC), and 100-fold dilution (DHQC) – each with 5 replicates on each of 4 different validation runs (n=20). Accuracy (% DEV) was defined as the percent difference between the mean observed concentration and the nominal concentration. Precision (% CV) was defined as the quotient of the standard deviation of the mean observed concentration and the mean observed concentration. The repeatability of the assay was determined by the within-run precision (WRP) and between-run (BRP), as calculated below:

$$WRP = 100 \times [(MS_{WIT})^{0.5}/GM] \quad \text{Equation 1}$$

$$BRP = 100 \times [((MS_{BET} - MS_{WIT})/n)^{0.5}/GM] \quad \text{Equation 2}$$

GM represents the grand mean, MS_{wit} represents within-group mean squared, MS_{bet} represents between-group mean squared, and n represents the number of repetitions. FDA guidelines for bioanalytical validation were followed, with $\pm 15\%$ variability in accuracy and precision allowed except for the LLOQ, where $\pm 20\%$ variability is acceptable[13].

2.5.3. Limit of Detection—A limit of detection was established to elucidate the lowest quantifiable concentration using this procedure. A peak must maintain a signal to noise ratio of greater than 3 in order for the peak to be quantifiable. Samples were run alongside a blank and IS in quintuplet to ensure the signal achieved is from the analyte as opposed to crosstalk from the internal standards or background signal.

2.5.4. Matrix Effects and Extraction Efficiency—The extraction efficiency for CP and 4OHCP were evaluated by comparing the peak areas of normally extracted samples (n=5) to post-extraction spiked samples (n=5). A low (50 and 5 ng/mL for CP and 4OHCP) and a high (2500 and 250 ng/mL for CP and 4OHCP) concentration of both analytes were used in order to establish the extraction efficiency throughout the range of the assay.

Matrix (phenylhydrazine/plasma supernatant) effects were assessed by comparing the peak area of a post-extraction spiking procedure (n=5) to the same concentration spiked into clean mobile phase (n=3). The concentrations match those listed above.

Processing efficiency was evaluated by comparing the peak area of the clean mobile phase spike (n=3) to the peak area of the normal extraction procedure (n=5). Again, the concentrations were the same as above.

2.5.5. Stability—The blood and plasma stability of CP and 4OHCP have been previously studied[4]. Due to a small reconstitution volume of 50 uL, evaporation overnight prevents reinjection of samples for 24-hour autosampler stability tests of this particular procedure. To combat this, two sets of QCs were prepared side by side with one set injected immediately after prepping. The second set was covered to prevent evaporation then left in the autosampler at 4°C for 24 hours prior to injection. Bench top stability was assessed over 2 hours at room temperature at both high (8560 and 856.0 ng/mL for CP and 4OHCP, respectively) and low (171.2 and 17.12 ng/mL for CP and 4OHCP, respectively) concentrations. Stability during 3 freeze thaw cycles was assessed at the same concentrations listed above. Samples were frozen at –80°C and thawed at 4°C.

2.5.6. Clinical Application—Study samples were serially collected on a clinical trial where patients with sickle cell disease underwent haploidentical peripheral blood stem cell transplantation (clinicaltrials.gov identifier NCT00977691)[11]. Clinical applicability of this assay for CP and 4OHCP was ascertained by running the study samples alongside freshly prepared standards and QCs. CP was intravenously infused at a dose of 50 mg/kg over the course of 1 hour. Two doses were infused over 48 hours beginning at times t=0 and t=24. Samples were drawn at time points of 0, 0.5, 1, 4, 6, 12, 24, 25, and 48 hours from 10 subjects. The pharmacokinetics of CP and 4OHCP are well characterized and will not be focused on here, rather the resulting concentration vs time profiles will be presented as assay applicability.

2.5.7. Incurred Sample Reanalysis—For further validation of the reliability of study samples analyzed using the reported assay, incurred sample reanalysis (ISR) was performed according to FDA Guidelines for Bioanalytical Method Validation [13]. Briefly, samples were analyzed on separate days using freshly prepared standards and QCs. In total, ISR was performed for 70% of the 90 samples (n=63) to cover the full PK profile studied with the current assay. The percent difference for each sample between runs was calculated using the following equation:

$$(\text{Repeated sample} - \text{Original sample}) / \text{Mean} * 100 \quad \text{Equation 3}$$

The assay is considered reproducible if 67% of the analyzed samples have less than a 20% difference between initial and repeated runs.

3. Results

3.1. Selectivity

Figure 2 depicts the chromatograms of drug-free supernatant, internal standard-only supernatant, an LLOQ sample with IS, and an undiluted study sample with IS drawn 24 hours after infusion start. The retention times for both CP and its internal standard, d4-CP, were 0.93 minutes. The retention times for 4OHCP and its internal standard, AZD7451, were 1.23 minutes and 1.30 minutes, respectively. Peaks for both analytes and internal standards were well defined from the surrounding background. The large, wide peaks eluting at 0.5 minutes are a result of the diverter valve shifting from waste to injection into the mass spectrometer.

3.2. Linearity, Accuracy, and Precision

Each calibration standard was run in duplicate on 4 days (n=8) with a range of 34.24–34,240 ng/mL and 3.424–3,424 ng/mL for CP and 4OHCP, respectively. Both calibration curves are precise and accurate within 15% (Table 1). The CP curve had a mean linear correlation (r) of 0.9960 ± 0.0013 (n=4), and the 4OHCP had a mean linear correlation of 0.9967 ± 0.0004 (n=4).

For each compound, 6 quality control samples were run, LLOQ (34.24 and 3.424 ng/mL), LQC (102.72 and 10.272 ng/mL), MQC (5,136 and 513.6 ng/mL), HQC (30,816 and 3,081.6 ng/mL), DQC (85,600 and 8,560 ng/mL), and DHQC (684,800 and 68,480 ng/mL). Each QC was run in quintuplet for 4 days (n=20). All of the QCs were accurate and precise within 15% as per FDA guidelines (Table 2). The %BRP cannot be calculated for the LQC of both CP or 4OHCP because in Equation 2 $MS_{BET} < MS_{WIT}$. Given this relationship, the square root of the difference between MS_{BET} and MS_{WIT} would give an imaginary number, therefore, it is not reported.

3.3. Limit of Detection

Because 4OHCP is the transport metabolite that provides information about the active metabolite within the cell, its LOD is of greater interest than that of CP. The LOD of 4OHCP was determined to be 0.856 ng/mL with a signal to noise ratio (S:N) of 6.3 in plasma. Further dilution down to 0.3424 ng/mL had a S:N of 2.1, deeming it undetectable. CP was detectable down to the lowest measured concentration of 3.424 ng/mL with a S:N of 19.2. Because of the high S:N, it is feasible that a lower LOD could be established for CP, however, it was not necessary for the scope of this assay or the clinical trial for which it has been developed.

3.4. Matrix Effects and Extraction Efficiency

Matrix effects and extraction efficiency were tested using three different CP/4OHCP stock solutions. Initially, the effects were studied using the original stock solution solvent – the phenylhydrazine solution for derivatization. This proved difficult because of the presence of

concentrated salts when spiked into the mobile phase, and it yielded unintuitive results, namely much greater signal area for both analytes when extracted from the usual supernatant versus spiked clean into the reconstitution solution. To remove this effect, stocks were prepared in ACN. The effect lessened, but persisted in the more concentrated stock because the salts from the 4OHCP master stock had yet to be diluted to near imperceptible levels, as was the case in the less concentrated stock.

Therefore, as a surrogate of the stocks used in the assay, stocks of only CP were diluted in ACN. Using this strategy, CP matrix effects, extraction efficiency, and recovery could be studied. For the low concentration samples (50 ng/mL) the matrix effect was 6.68%, the process efficiency was 10.83%, and the recovery 162.14%. The high concentration samples (2500 ng/mL) had a matrix effect of -80.3% due to ion suppression, an extraction efficiency of 115%, and an overall process efficiency of 22.8%.

3.5. Stability

The stability of CP and 4OHCP was assessed in 4:3 phenylhydrazine solution:whole blood supernatant. Low and high concentrations were run in triplicate for 2-hour bench top stability and freeze thaw stability (Table 3A). CP had <15% change in concentration after 3 freeze thaw cycles, just as previously reported[12]. Similarly, <5% change in average concentration of CP occurred when left at room temperature for 2 hours prior to extraction. A 20% and 30% decrease in 4OHCP concentration occurred for bench top stability and freeze thaw stability (Table 3B). Therefore, samples should be handled on ice at all times to minimize this effect. Autosampler stability was assessed over a 24-hour time period using two sets of QCs prepared simultaneously. CP showed very good stability with <3% change in concentration over 24 hours. 4OHCP concentration was unstable in the autosampler with >15% change over 24 hours.

3.6. Clinical Application

The average plasma concentration of both CP and 4OHCP at each time point for 10 patients given a daily 1-hour intravenous infusion is shown in Figure 3. From these initial data, it is apparent that clinical samples from relevant doses can be measured using this assay. The LLOQ also is sufficient to measure all clinically-relevant concentrations of both parent and metabolite. Further pharmacokinetic analysis will be published upon completion of this ongoing clinical trial.

3.7. Incurred Sample Reanalysis

ISR was performed on 63 samples for both CP and 4OHCP. Both analytes showed excellent reproducibility. Of the 63 reanalyzed samples, 52 (82.54%) and 53 (84.13%) had less than a 20% difference between the first and second runs. Moreover, the average percent deviation for all 90 samples was 14.42% and 19.88% for CP and 4OHCP, respectively.

4. Conclusion

LC-MS/MS assays have been published for CP and 4OHCP prior to this paper. However, this paper describes the first time that both CP and 4OHCP were measured during the same

analytical run with such high sensitivity. Using a simple MTBE liquid-liquid extraction that affords quick sample preparation, a high level of accuracy and precision was achieved down to the LLOQ of 34.24 and 3.424 ng/mL for CP and 4OHCP, respectively. Subsequently, this assay is ideal for pharmacokinetic studies of CP and 4OHCP that requires only a single blood draw. This eases the burden on both the patient and clinical staff, alike.

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Abbreviations

CP	Cyclophosphamide
4OHCP	4-hydroxycyclophosphamide
d4-CP	deuterated cyclophosphamide
FA	formic acid
IS	internal standard
LOD	limit of detection

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Highlights

- A rapid and accurate LCMSMS assay to simultaneously quantify cyclophosphamide and 4-hydroxycyclophosphamide.
- Lowest limit of quantitation for 4-hydroxycyclophosphamide to date.
- Demonstrated clinical applicability of assay for both analytes.

Figure 1.

shows each of the analytes and internal standards utilized in the reported assay. In the first row are the analyte cyclophosphamide (left) and its internal standard d4-cyclophosphamide (right). The second row features 4-hydroperoxycyclophosphamide (left) and 4-hydroxycyclophosphamide (right), which is formed upon spontaneous reduction of the peroxy-form (left) in water. On the bottom row is AZD7451, the internal standard for 4-hydroxycyclophosphamide.

Figure 2.

shows typical chromatograms of samples run using the reported assay. The MRM monitored 4 sets of mass transitions – AZD7451, 4-hydroxycyclophosphamide, d4-cyclophosphamide, and cyclophosphamide from top to bottom. In panel (A), the chromatogram depicts a blank plasma sample extracted with pure MTBE. Panel (B) shows blank plasma extracted with IS (first and third peaks) in MTBE. Panel (C) is the lower limit of quantitation. And panel (D) is a typical patient sample.

Figure 3.

is a concentration vs time curve of both cyclophosphamide (blue) and 4-hydroxycyclophosphamide (red) in patient samples from an ongoing clinical trial. Over the 48-hour time period, two doses were given at $t=0$ hours and $t=24$ hours. The lower limit of quantitation is provided as the dotted lines where $y=34.24$ (cyclophosphamide) and $y=3.424$ (4-hydroxycyclophosphamide). Concentration is measured in ng/mL and depicted on a $\log_{10}$ scale.

Table 1

Calibration Linearity for CP (A) and 4OHCP (B).

(A)					
Nominal (ng/ml)	GM (ng/ml)	SD (ng/ml)	DEV (%)	CV (%)	n
34.24	35.81	1.89	4.58%	5.28%	8
68.48	64.45	4.78	-5.89%	7.42%	8
171.2	158.4	7.91	-7.46%	4.99%	8
856	799.8	33.44	-6.57%	4.18%	8
1712	1744	59.39	1.85%	3.41%	8
3424	3612	131.1	5.48%	3.63%	8
8560	9413	369.5	9.96%	3.93%	8
34240	33440	1291	-2.33%	3.86%	8

(B)					
Nominal (ng/ml)	GM (ng/ml)	SD (ng/ml)	DEV (%)	CV (%)	n
3.424	3.70	0.36	8.00%	9.64%	8
6.848	6.36	0.69	-7.13%	10.90%	8
17.12	16.86	1.38	-1.50%	8.17%	8
85.6	92.02	11.70	7.49%	12.71%	8
171.2	154.1	16.54	-9.97%	10.73%	8
342.4	331.9	20.40	-3.06%	6.15%	8
856	877.0	49.18	2.45%	5.61%	7
3424	3557	222.3	3.88%	6.25%	8

Abbreviations: GM, grand mean; SD, standard deviation; DEV (%), relative deviation from nominal value; CV (%), coefficient of variation; *n*, number of replicate observations within each validation run, i.e. two samples at each concentration were run on four separate occasions, for a total (*n*) of eight samples at each concentration.

Table 2

Accuracy and Precision for CP (A) and 4OHCP (B).

(A)						
Nominal (ng/ml)	GM (ng/ml)	SD (ng/ml)	DEV (%)	WRP (%)	BRP (%)	n
34.24 (LLOQ)	33.02	5.07	-3.57%	12.61	9.85	20
102.72 (LQC)	99.71	5.33	-2.93%	5.48	-	20
5136 (MQC)	5520	224.5	7.48%	3.62	2.08	20
30816 (HQC)	30150	798.6	-2.16%	2.11	1.81	20
85600 (DQC)	97350	3809	13.73%	3.20	1.87	20
684800 (DHQC)	755400	77860	10.31%	2.84	10.85	20

(B)						
Nominal (ng/ml)	GM (ng/ml)	SD (ng/ml)	DEV (%)	WRP (%)	BRP (%)	n
3.424 (LLOQ)	3.22	0.35	-6.00%	10.15	4.28	20
10.272 (LQC)	10.05	0.77	-2.18%	7.92	-	20
513.6 (MQC)	513.1	26.80	-0.09%	4.79	2.34	20
3081.6 (HQC)	3138.4	339.6	1.84%	4.75	10.94	20
8560 (DQC)	9324	1328	8.92%	9.42	12.03	20
68480 (DHQC)	71310	8193	4.14%	4.47	11.91	20

Abbreviations: GM, grand mean; SD, standard deviation; DEV (%) relative deviation from nominal value; WRP, within-run precision; BRP, between-run precision; n, number of replicate observations within each validation run. (A hyphen indicates $MS_{\text{W}} > MS_{\text{B}}$, thus BRP cannot be calculated and it was concluded that no additional variation was observed as a result of performing the assay in different runs)

Table 3

Bench Top and Freeze Thaw Stability for CP (A) and 4OHCP (B).

(A)						
Fresh						
	Predicted Concentration (ng/mL)	GM (ng/mL)	Dev (%)	SD	CV (%)	% Change
Low	171.2	173.3	1.20	1.84	1.06	-
High	8560	9166	7.08	326.27	3.56	-
2-hr Bench Top						
	Predicted Concentration (ng/mL)	GM (ng/mL)	Dev (%)	SD	CV (%)	% Change
Low	171.2	168.8	-1.42	3.03	1.79	-2.59
High	8560	8818	3.01	531.57	6.03	-3.8
Freeze Thaw (3 cycles)						
	Predicted Concentration (ng/mL)	GM (ng/mL)	Dev (%)	SD	CV (%)	% Change
	(ng/mL)					
Low	171.2	174.4	1.86	6.06	3.47	0.65
High	8560	8181	-4.42	337.46	4.12	-10.74

(B)						
Fresh						
	Predicted Concentration (ng/mL)	GM (ng/mL)	Dev (%)	SD	CV (%)	% Change
Low	17.12	18.88	10.30	3.41	18.08	-
High	856.0	931.7	8.84	13.82	1.48	-
2-hr Bench Top						
	Predicted Concentration (ng/mL)	Mean (ng/mL)	Dev (%)	SD	CV (%)	% Change
Low	17.12	15.76	-7.93	1.18	7.48	-16.53
High	856.0	737.4	-13.85	42.67	5.79	-20.85
Freeze Thaw (3 cycles)						
	Predicted Concentration (ng/mL)	Mean (ng/mL)	Dev (%)	SD	CV (%)	% Change
Low	17.12	13.24	-22.67	0.78	5.92	-29.89
High	856.0	658.1	-23.12	31.97	4.86	-29.37

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Abbreviations: GM, grand mean; SD, standard deviation; DEV (%) relative deviation from Predicted Concentration; % CV, coefficient of variation.