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Short communication

Simultaneous quantification of cyclophosphamide and its active metabolite 4-hydroxycyclophosphamide in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC–MS/MS)

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Abstract

Cyclophosphamide is a cytotoxic prodrug with a very narrow therapeutic index. To study the clinical pharmacology of cyclophosphamide in a large cohort of patients a previously published method for the simultaneous quantitative determination of cyclophosphamide and 4-hydroxycyclophosphamide in human plasma using liquid chromatography tandem mass spectrometry (LC–MS/MS) was optimized. Addition of an isotopically labelled internal standard and adaptation of the gradient resulted in a fast, robust and sensitive assay. Because 4-hydroxycyclophosphamide is not stable in plasma, the compound is derivatized with semicarbazide immediately after sample collection. Sample preparation was carried out by protein precipitation with methanol–acetonitrile (1:1, v/v), containing isotopically labelled cyclophosphamide and hexamethylphosphoramide as internal standards. The LC separation was performed on a Zorbax Extend C18 column (150 mm × 2.1 mm ID, particle size 5 μm) with 1 mM ammonium hydroxide in water–acetonitrile (90:10, v/v) as the starting gradient, at a flow-rate of 0.40 mL/min with a total run time of 6 min. The lower limit of quantification (LLQ, using a 100 μL sample volume) was 200 ng/mL and the linear dynamic range extended to 40,000 ng/mL for cyclophosphamide and 50–5000 ng/mL for 4-hydroxycyclophosphamide. Accuracies as well as precisions were lower than 20% at the LLQ concentration and lower than 15% for all other concentrations. This method has been successfully applied in our institute to support ongoing studies into the pharmacokinetics and pharmacogenetics of cyclophosphamide.

Keywords: LC–MS/MS; Cyclophosphamide; 4-Hydroxycyclophosphamide

1. Introduction

Cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide; CP) is a cytotoxic drug widely used in the treatment of various malignancies. It is a prodrug that requires activation by the cytochrome P450 enzyme system (CYP) to form its pharmacologically active metabolite 4-hydroxycyclophosphamide (4OHCP) [1].

Assays quantifying CP and/or 4OHCP using high-performance liquid chromatography (HPLC) with mass spectrometry (MS) detection have been described previously [2–6]. However, these methods either described the quantification of CP and other metabolites in urine [2,3] or quantification of 4OHCP without CP in human plasma [4]. Sadagopan et al.

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described the simultaneous quantification of CP and 4OHCP in mouse plasma [5]. Baumann et al. described the simultaneous quantification of CP and 4OHCP in human plasma albeit with a run time of 20 min [6]. In an earlier publication we described the development and validation of a liquid chromatography tandem mass spectrometric assay for the simultaneous quantification of CP, 4OHCP, thiotepa and tepa in human plasma [7]. This assay has been used successfully for the therapeutic drug monitoring (TDM) of CP and thiotepa [8]. However, application of this assay in a large-scale pharmacokinetic study of cyclophosphamide was limited by the run-time and the absence of a good internal standard for cyclophosphamide.

In this paper, we present an optimized method for the simultaneous determination of CP and 4OHCP in human plasma using HPLC coupled with electrospray ionization (ESI) MS/MS with a run time of 6 min, using a stable isotope for the quantification of CP to improve the accuracy and precision of the method. A partial validation of the adapted method was performed based on the international FDA guidelines for bioanalytical validation [9]. Stability data have been reported before [7].

2. Experimental

2.1. Chemicals

CP and 4-hydroperoxycyclophosphamide (4OOHCP) and d4-cyclophosphamide (d4-CP) were purchased from Niomech, Bielefeld, Germany (purity > 95%). Hexamethylphosphoramide (HMP) originated from Sigma (Zwijndrecht, The Netherlands).

Acetonitrile and methanol were HPLC-grade reagents and were obtained from Biosolve BV (Valkenswaard, The Netherlands). Semicarbazide hydrochloride (analytical reagent grade) was purchased from Acros (Geel, Belgium). Distilled water (B. Braun Medical, Emmenbrücke, Switzerland) was used throughout the analysis and all other chemicals used were of analytical grade and used without further purification. Drug-free human heparinized plasma originated from Bioreclamations, Hicksville, New York, USA.

2.2. Instrumentation

The LC system consisted of an Agilent 1100 series pump, degasser and cooled autosampler (10 °C) (Agilent Technologies). A stepwise gradient was used to elute the compounds from a Zorbax Extend C18 analytical column (150 mm × 2.1 mm ID, particle size 5 μm; Agilent Technologies, Palo Alto, CA, USA) protected with an Agilent Extend C18 Narrow-Bore Guard Column (12.5 mm × 2.1 mm ID, particle size 5 μm; Agilent Technologies). At time zero, 90% eluent A (1 mM ammonium hydroxide in water, pH 10) and 10% eluent B (100% acetonitrile) was flushed through the column. After 0.5 min, the fraction of acetonitrile was increased to 35% in 0.01-min time. This mobile-phase composition was maintained for 1.5 min. Subsequently, in 0.01-min time the mobile-phase composition was set back at 90% eluent A, remaining as such for the final 4 min of the run. The flow rate was 0.4 mL/min. The eluate (first two minutes of run discarded) entered into an API 3000 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) controlled by Analyst™ software (Applied Biosystems/MDS Sciex, Analyst software version 1.2). The source temperature was set at 400 °C. The curtain gas (1.1 ml/min) and the collision-induced dissociation gas (342×10^{15} molecules/cm^2) consisted of nitrogen (grade 5.0) and the nebulizer and turbo gases (1.6 l/min and 7.0 l/min, respectively) were zero air. The electrospray source was operated in the positive ion mode. The electrospray voltage was +2.5 kV and the dwell time was 150 ms for HMP, 350 ms for CP and d4-CP and 400 ms for 4OHCP-semicarbazide with a 5 ms pause between scans. Mass transitions monitored were *m/z* 261 → 140 for CP, *m/z* 334 → 221 for 4OHCP-semicarbazide, *m/z* 267 → 146 for d4-CP and *m/z* 180 → 135 for HMP and were monitored in the positive multiple reaction monitoring (MRM) mode.

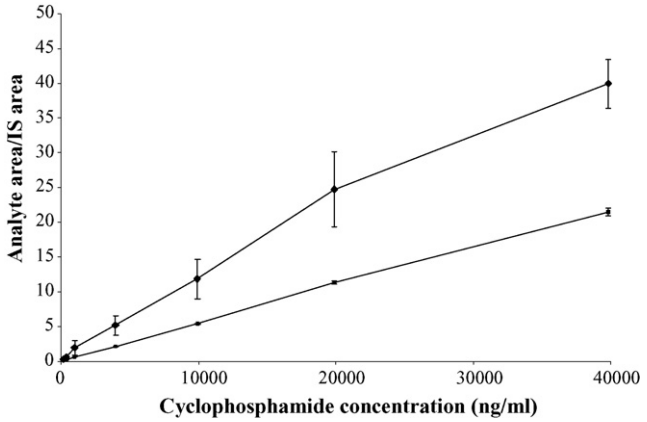

Fig. 1. Calibration curve of cyclophosphamide using hexamethylphosphoramide(♦) and d4-cyclophosphamide(■) as internal standard.

2.3. Sample preparation

Since 4OHCP is not stable in plasma, but degrades rapidly into phosphoramide mustard, semicarbazide was used for stabilization of 4OHCP, and quantified as the 4OHCP-semicarbazide derivative, a reaction optimised previously [10]. To 100 μL plasma sample derivatized with semicarbazide, 25 μL of IS solution I (20 ng/mL d4-CP) for the quantification of CP and 25 μl

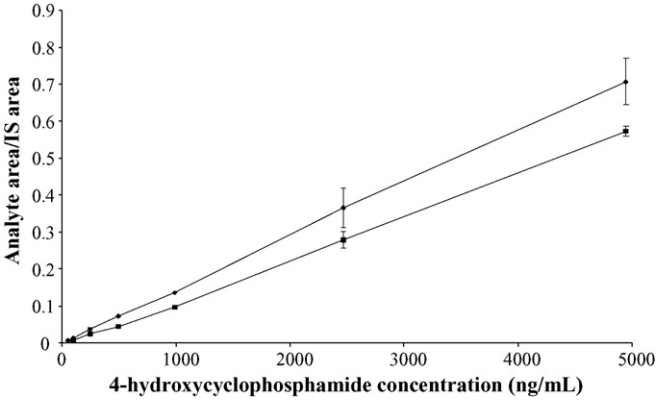

Fig. 2. Calibration curve of 4-hydroxycyclophosphamide using hexamethylphosphoramide(♦) and d4-cyclophosphamide(■) as internal standard.

of IS solution II (100 ng/mL HMP) for the quantification of 4OHCP-semicarbazide were added. Protein precipitation was performed by addition of 300 μL methanol–acetonitrile (1:1, v/v). The samples were mixed and centrifuged for 15 min at 23,100 × *g*. After dilution of 50 μL of supernatant with 400 μL eluent A (1 mM ammonium hydroxide in water), 10 μL aliquots were injected onto the analytical column.

2.4. Validation procedures

A partial validation based on the FDA guidelines was performed by means of linearity, accuracy and precision, selectivity and specificity, cross-analyte/IS interference and carry-over. Furthermore, clinical application of the assay was demonstrated.

3. Results

3.1. Chromatography

LC separation of the two analytes was performed under basic conditions using an eluent composed of aqueous 1 mM ammonium hydroxide and acetonitrile together with a column containing a base-stable stationary phase (pH of the aqueous component was 10). The capacity factors for CP and 4OHCP-semicarbazide were 2.1 and 1.5, respectively. The run time could be limited to 6 min. For the quantification of CP the deuterated IS d4-CP resulted in intra-assay imprecisions of less than 2.77% versus 26.3% when HMP was used as IS. Furthermore, the calibration curve of CP was linear over the complete validated range in contrast to the curve of CP with HMP as internal standard (Fig. 1). 4OHCP-semicarbazide could be best quantified using HMP as IS, probably due to the same retention time of 4OHCP-semicarbazide and HMP. Furthermore, when d4-CP was used as IS for the quantification of 4OHCP-semicarbazide the LLQ of 50 ng/mL could not be quantified with sufficient accuracy and precision and needed to be increased to 100 ng/mL. The calibration curve of 4OHCP with HMP and d4-CP as internal standards is shown in Fig. 2.

3.2. Validation procedures

3.2.1. Linearity

For the validation, calibration standards (seven non-zero standards) were prepared in control human plasma and analyzed in duplicate in three analytical runs. The assay was linear over the validated concentration ranges of 200–40,000 ng/mL for CP and 50–5,000 ng/mL for 4OHCP.The best fit for the calibration curves was obtained by using a weighting factor of 1/concentration^2 for CP and 1/concentration for 4-OHCP. Correlation coefficients were >0.995. At all concentration levels deviations of measured concentrations from nominal concentrations were between −3.27 and 4.02% with CV values less

Table 1
Inter-assay performance data for cyclophosphamide and 4-hydroxycyclophosphamide

Compound	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Inter-assay inaccuracy (%)	Inter-assay inaccuracy (HMP as IS)[a] (%)	Inter-assay imprecision (%)	Inter-assay imprecision (HMP as IS)[a] (%)	Number of replicates
Cyclophosphamide	199	199	−0.067	−3.74	0.907	9.79	15
	398	390	−2.08	−0.79	2.16	3.64	15
	3980	3971	−0.235	7.15	1.26	3.22	15
	19900	20093	0.972	3.84	1.55	2.13	15
4-Hydroxy-cyclophosphamide	50	54	7.91		8.43		15
	102	96	−5.53		3.69		15
	508	501	−1.34		7.08		15
	2540	2525	−0.577		2.06		15

[a] Assay performance data of previous method [7].

Table 2
Intra-assay performance data for cyclophosphamide and 4-hydroxycyclophosphamide

Compound	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Intra-assay inaccuracy (%)	Intra-assay imprecision (%)	Intra-assay imprecision (HMP as IS)[a] (%)	Number of replicates
Cyclophosphamide	199	197	−1.01	2.38	6.32	5
	398	398	0.00	1.38	2.73	5
	3980	3958	−0.553	1.42	2.75	5
	19900	19840	−0.302	2.77	1.94	5
4-Hydroxy-cyclophosphamide	50	56	13.7	7.08		5
	102	94	−7.73	5.59		5
	508	504	−0.787	3.17		5
	2540	2514	−1.02	3.18		5

[a] Assay performance data of previous method [7].

than 4.46% for CP. For 4OHCP deviations of measured concentrations from nominal concentrations were between −3.13 and 5.10% with CV values less than 9.65%.

3.2.2. Accuracy and precision

Five replicates of independently prepared quality control samples in human plasma (200, 400, 4000 and 20,000 ng/mL of CP and 50, 100, 500 and 2500 ng/mL of 4OHCP) were analyzed in three analytical runs together with calibration standards. Intra-assay inaccuracy was determined as the percent difference between the mean concentration in one analytical run and the nominal concentration, inter-assay inaccuracy as the percent difference between the mean concentration after three analytical runs and the nominal concentration. Intra-assay imprecision was determined as the standard deviation in an analytical run divided by the mean of the run. Inter-assay imprecision was determined as the standard deviation of three analytical runs divided by the overall mean. Assay performance data for CP and 4OHCP are summarized in Tables 1 and 2. The assay performance data of the previous method [7] for CP are also included in Tables 1 and 2.

Samples originally above the upper limit of quantification (ULQ) could be quantified with acceptable accuracy and precision after dilution with drug-free human plasma. Measured inaccuracies ranged from 1.85% to 3.87% for CP and 0.921% to 5.39% for 4OHCP. Imprecisions ranged from 1.00% to 2.90% for CP and 2.45% to 6.34% for 4OHCP.

As defined by the lower and upper validation sample concentrations possessing acceptable accuracy and precision, the validated range of this method, based on 100 μL of derivatized human plasma, is 200–40,000 ng/mL for CP and 50–5,000 ng/mL for 4OHCP (measured as 4OHCP-semicarbazide). This range was suitable for use in our clinical studies, as demonstrated by quantification of plasma samples obtained from a patient treated with CP (Fig. 3).

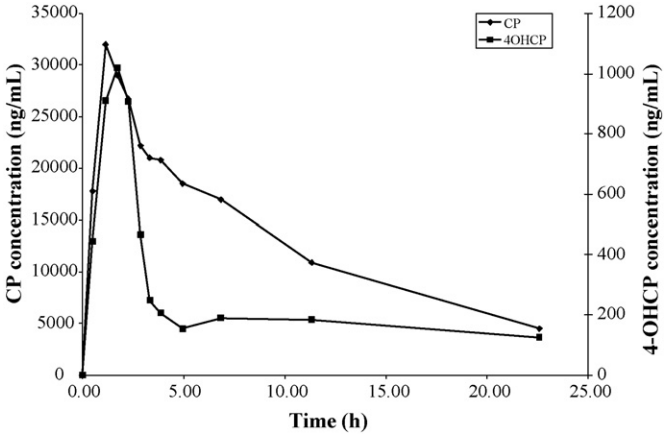

Fig. 3. Concentration–time curves of cyclophosphamide and 4-hydroxycyclophosphamide of a patient receiving a 1-h infusion of 1500 mg cyclophosphamide.

3.2.3. Selectivity and specificity

MRM chromatograms of six batches of control human plasma contained no co-eluting peaks >20% of the CP and 4OHCP-semicarbazide areas at the LLQ level, and no co-eluting

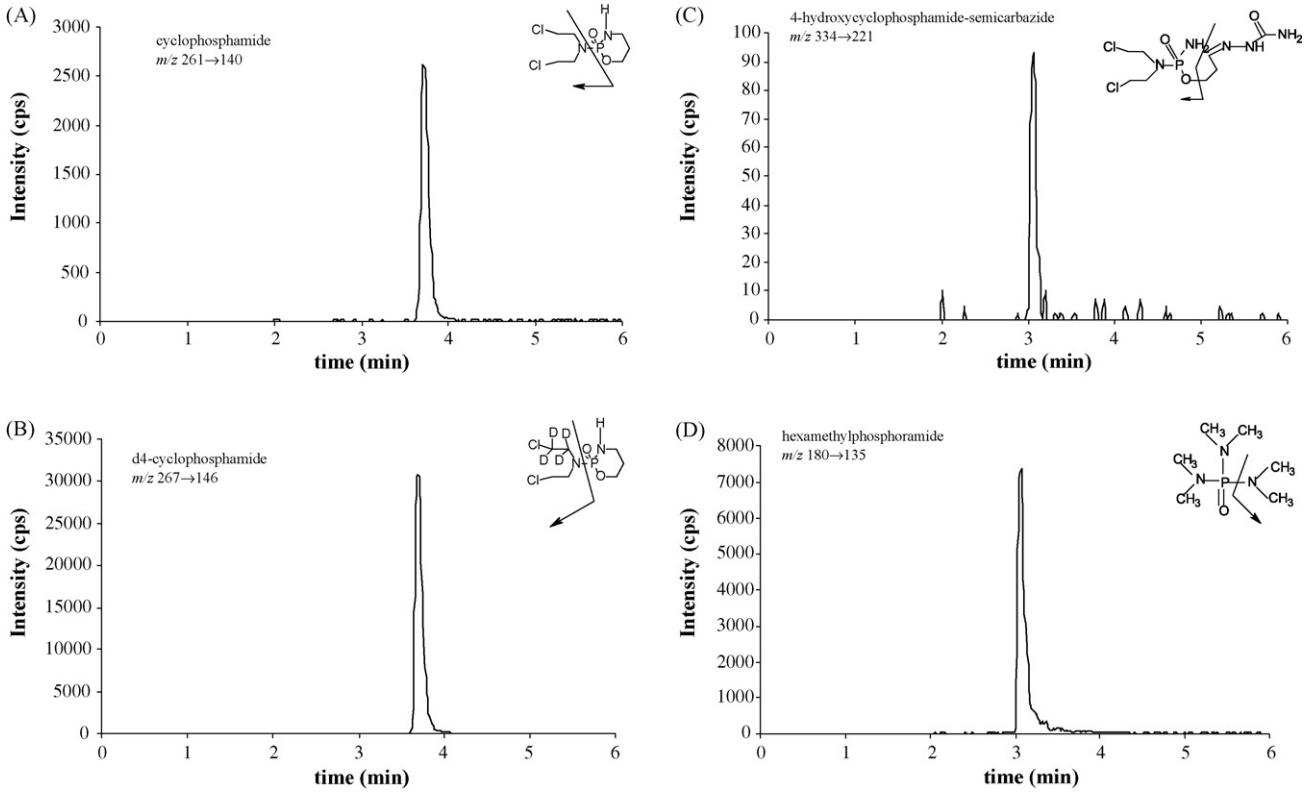

Fig. 4. MRM chromatograms (A–D) for a processed sample at the lower limit of quantification level. Cyclophosphamide (A, 200 ng/mL); d4-cyclophosphamide (B, 5 ng/mL); 4-hydroxycyclophosphamide as a semicarbazone derivative (C, 50 ng/mL); hexamethylphosphoramide (D, 25 ng/mL).

peaks >5% of the area of the IS. Fig. 4 shows MRM chromatograms of the analytes and IS. Deviations from the nominal concentrations at the LLQ level were between −9.55% and 0.00% for CP and between −20.0% and 3.03% for 4OHCP and thus acceptable.

3.3. Cross-analyte/internal standard interference

Four separate blank plasma samples were spiked with CP at the ULQ level, 4OHCP at the ULQ level, d4-CP and HMP to determine whether these compounds show interference with each other. No interference from cross-analytes and IS at the retention time of CP and 4OHCP-semicarbazide with areas >20% of the LLQ area, nor interference with areas >5% of the IS area were observed.

3.3.1. Carry-over

To determine the carry-over, two blank samples were injected after a sample at the ULQ level (containing CP, 4OHCP-semicarbazide, d4-CP and HMP). No carry-over from the analytes and IS in the first blank sample after the sample at the ULQ level with an area >20% of the corresponding peak area at the LLQ level was observed.

4. Conclusion

For the simultaneous quantification of CP and its active metabolite 4OHCP (measured as 4OHCP-semicarbazide) in human plasma, a fast, accurate, reproducible and selective LC–MS/MS assay has been developed. The assay quantifies a range for CP from 200 to 40,000 ng/mL and for 4OHCP from 50 to 5,000 ng/mL, sufficient for pharmacokinetic studies with cyclophosphamide in both low and high-dose regimens, using 100 μL human plasma aliquots. Validation results demonstrate that CP can be accurately quantified using d4-CP as IS and 4OHCP concentrations (as 4OHCP-semicarbazide) can be accurately quantified using HMP as IS. Imprecision was markedly reduced by using a deuterated IS. This assay with a runtime of 6 min is now used to support ongoing studies into the pharmacokinetics and pharmacogenetics of CP and TDM programs with CP in our institute.

Acknowledgement

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