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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 min), 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 (Cmax 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 3.424–3424 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.

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, phosphoramide 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, Ekhart 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

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

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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 min. 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 uL versus 500 uL. Furthermore, the extraction procedure has been streamed lined 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 ($^2[H_4]$) 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 Fig. 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 µg/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 min to achieve maximum derivatization [2,11].

Stock solutions were prepared from the 1 mg/mL master stock of CP, and the 100 µg/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, 1712, 8560, 17,120, 34,240, 85,600, and 342,400 ng/mL for CP; and 34.24, 684.8, 171.2, 856, 1720, 3240, 8560, 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 min at 2000 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 3.424–3424 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, 5136, and 30,816 ng/mL and 10.272, 513.6, 3081.6 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 8560 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 uL of sample supernatent, 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 min 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 min 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 s to re-suspend analyte residue, followed by centrifugation for 10 min 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 µm 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 H_2O/ACN/FA [2], with a flow rate of 0.35 mL/min was maintained for the entire 3 min 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 min of the run. It was then switched to injecting into the mass spectrometer until 1.5 min 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