Development and validation of a sensitive and specific hplc assay of cladribine for pharmacokinetics studies in rats *

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ABSTRACT - Purpose: To develop and validate a sensitive and specific HPLC assay for cladribine (CdA) in plasma for pharmacokinetic studies in rats. Methods: CdA and the internal standard AZT were purchased from Sigma-Aldrich Chem. The HPLC system consisted of a Shimadzu LC-9A pump, a 3 μm, 250 x 2.0 mm I.D. high speed C 18 column (Jupiter®), preceded by a 5 μm 4 x 4 mm I.D. C 18 guard column (Licrocart®), an Agilent Model 1050 UV-VIS detector and a 3395 Integrator. The mobile phase was made up of 0.01M KH2PO4 (pH 5): methanol: acetonitrile (90:5:5). The system was operated at ambient temperature with a flow rate of 0.3 mL/min, and UV wavelength at 265 nm, and an operating pressure of ~ 1.56 kpsi. Extraction of cladribine and AZT from plasma was achieved by solid phase extraction using 100 mg/mL C18 SPE columns (Extra-sep®). The assay was validated for sensitivity, precision, specificity and application for pharmacokinetic study in rats. Results: Under these conditions, the average retention times of CdA and AZT were 13.5 and 21 min, respectively, and recoveries were between 80 – 95%. Standard curve constructed from plasma standards was linear from 0.1 μg/mL to 1 μg/mL with regression coefficient ($r^2$) 0.99 or greater. Sensitivity assessed by on-column injection was < 1 ng. Using a 50-uL plasma sample size, the mean intra-assay variations at 0.1 μg/mL were 7%, and inter-assay variations over a period of 3 months for 5 separate batches were less than 20%. The assay was used to study a single dose pharmacokinetic study of CdA in rats after a 2 mg/kg subcutaneous injection. Conclusion: The described HPLC assay has adequate sensitivity and specificity to study pharmacokinetics of CdA in rats, and could be adapted also to clinical pharmacokinetic studies.

INTRODUCTION

Cladribine (2-chlorodeoxyadenosine, CdA) has been shown to be highly effective for treatment of several haematological malignancies, including hairy cell leukaemia and chronic lymphocytic leukemia (CLL) (1), and could have considerable potential also for solid tumors and autoimmune diseases (2-4). CdA is a deoxypurine nucleoside analogue that resists deamination by adenosine deaminase (1). It is phosphorylated intra-cellularly by deoxycytidine kinase to 2-chlorodeoxyadenosine-5'-triphosphate, which gets incorporated into DNA, and inhibits cancer progression and subsequently leads to cell death (5). CdA is currently recommended to be administered as a 7-day intravenous infusion (either continuous or 2 h/day) (3). This mode of drug administration requires specialized staffs and is inconvenient for the patient. Also, many adverse experiences (pain, thrombosis, phlebitis) are directly related to this IV injection method (6). Understanding the pharmacokinetics of CdA in plasma could help to better design dosage and route of administration for an optimum therapy.

Several methods have been described to measure CdA in plasma (7, 8). These methods used deproteinization with perchloric acid or solid phase extraction using C8 extraction columns. The extracts were analysed using reversed phase or ion-paired chromatography with ultraviolet (UV) detection at 265 nm. The current paper describes development and validation of a new HPLC assay for measuring plasma CdA concentration, which is simple, sensitive, and specific for measuring CdA using commercially available reagents, and applies it to a single dose pharmacokinetic study in rats.

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METHODS

**Chemicals**

CdA was purchased from Calbiochem (CA, USA), and AZT was purchased from Sigma-Aldrich Ltd (MO, Canada). Other pharmaceutical products tested for interference with the assay were either received as gifts from their respective manufacturers, or purchased from Sigma-Aldrich Ltd. Solid phase extraction (SPE) columns were C\textsubscript{18} materials (100 mg/mL) purchased commercially (Extra-Sep®, Chromatographic Specialties Inc., Brockville/ON, Canada.). Solvents were HPLC grade (BDH Chem., Halifax, N.S., Canada), and all other chemicals were reagent grade (Fisher Scientific, Ont., Canada).

**HPLC System**

The HPLC system consisted of a Shimadzu LC-9A solvent delivery system, a Beckman Model 210 switching valve injector, and a Hewlett-Packard Model 1050 variable UV-VIS spectrophotometric detector. Chromatographic separation was achieved on a 3 \mu m minibore 250 x 2.0 mm I.D high speed C\textsubscript{18} column, (Jupiter®, Phenomenex, CA, USA), preceded with a 4 x 4 mm C\textsubscript{18} guard column (Lichrocart®, EM Merck, Germany) using a mobile Phase of a mixture of 0.01 M potassium phosphate buffer at pH 5.0 (KH\textsubscript{2}PO\textsubscript{4}); methanol: acetonitrile (90:5:5), at room temperature with a flow rate of 0.3 mL/min and operating pressure of 1.10 kgf/cm\textsuperscript{2} (1.5 kpsi). CdA and AZT were detected and quantified at 265 nm. Detector output was recorded by an integrator (Hewlett-Packard HP3395 Integrator/Palo Alto, CA, USA), and digitalized using the Peak Simple® software (Chromatographic Specialties Inc., Brockville/ON, Canada).

**Preparation of standard Solutions**

Stock solutions of CdA and AZT were prepared at 0.1 mg/mL in methanol. Serial dilution of the stock solutions using 0.01 M phosphate buffer (pH 6.5) was performed to prepare standard working solutions of 1, 0.75, 0.5, 0.25, 0.1 and 0.05 ug/mL. Working internal standard solution of 1 ug/mL was prepared by diluting the stock AZT solution with the same phosphate buffer (0.01 M, pH 6.5).

**Solid Phase Extraction**

Fifty \mu l of 1 \mu g/mL AZT (50 ng), 50 \mu l of blank rat plasma or plasma sample, and 100 \mu l of 0.01 M KH\textsubscript{2}PO\textsubscript{4} at pH 6.5, were applied from the top of each of the 100 mg/mL C\textsubscript{18} extraction cartridges (Extra-Sep®), which were pre-washed with methanol followed by PBS prior to use. Fifty \mu L of a standard solution (1 \mu g/mL, 0.75 \mu g/mL, 0.50 \mu g/mL, 0.25 \mu g/mL, 0.10 \mu g/mL, and 0.05 ug/mL) of CdA in 0.01 M KH\textsubscript{2}PO\textsubscript{4} (pH 6.5) were added onto each cartridge. The mixtures were left to equilibrate at the top of the SPE column for 5 min and then passed through the columns slowly (ca. 1 mL/5 min) at a pressure of 10 inches of Hg. It was washed with 2 x 1 mL of distilled water and then air dried with maximum vacuum at 30 inches of Hg for 30 min. Each SPE column was washed with 2 x 1 mL of hexane:dichloromethane (1:1), and CdA and the internal standard AZT were desorbed by 10% methanol in ethyl acetate, and then collected into a cone shaped glass culture tube. The collected filtrate was evaporated to dryness under a gentle stream of nitrogen at 45 °C. The residues were reconstituted in 200 \mu L of mobile phase immediately prior to injection, and an aliquot (10 – 50 \mu L) was injected into the HPLC. Recoveries of CdA and AZT were determined by measuring the
amounts (expressed as peak heights) after the SPE extraction, and compared them to the amounts added to the quality control (QC) samples.

Pharmacokinetics Study

The study protocol was approved by the Dalhousie University Committee on Laboratory Animals (UCLA) using the Canadian Council of Animal Care (CCAC) guidelines. Male SD rats (350 – 450 g) purchased from Charles River Laboratories were each (n = 6) injected subcutaneously with a single dose of CdA (2 mg/kg). Blood samples (0.3 mL each) were obtained from the carotid artery catheter serially at 0.3, 0.5, 1, 2, 3, 4, 5 and 6 hours post dose. The plasma (> 0.1 mL) was immediately separated by centrifugation (4 °C, 1720 x g, 5 min) and then stored at –80 °C until analysis. An aliquot of the plasma samples (50 uL) was used for analysis of CdA by the described HPLC. All of the samples were analyzed within 3 months after collection.

Data analysis

Standard curves were plotted using known plasma concentrations of CdA (x-axis) and the peak height ratios of CdA to the internal standard AZT (y-axis), from 0.05 to 1 ug/mL and analyzed by linear regression (Lotus 1-2-3, IBM Canada). The QC samples at each concentration were performed in 4 replicates. Intra- and inter-assay variations were assessed from the QC samples (1 and 0.1 ug/mL) for each batch analysis over a 3-month period. Sensitivity of the assay was assessed by determining the smallest amount of analyte injected on-column which results in a signal to noise ratio of greater than 3. The lower limit of quantitation (LLQ) of the assay was assessed by the lowest concentrations of CdA measurable with a CV of less than 15%. Intra- and inter-assay variations were assessed using quality control samples at high (1 ug/mL) and low (0.1 ug/mL) over a 3 month-period over 5 separate batches of analyses. Pharmacokinetic variables such as area under the curve (AUC), maximum plasma concentration (Cmax), terminal half-life (t½ ß), and volume of distribution at steady-state (Vdss) were calculated using WinNonLin® (V. 5.01, Pharsight Corp., Mountain View, CA, USA) assuming a 2-compartment open model after an iv-bolus injection. The mean plasma concentration-time data from rats (n = 6) were used for calculating the pharmacokinetic parameter estimates.

RESULTS

Under the described chromatographic conditions, the average retention times of CdA, and AZT were 15 and 22 minutes, respectively (Figure 2). The standard curves constructed at different stages of the method development were linear between 0.05 to 1 ug/mL with regression coefficients of > 0.99 for CdA. A typical standard curve is shown in Figure 2. The recoveries of CdA using the described SPE were >90% for CdA at both 0.1 and 1 ug/mL, and also > 90% for AZT at the working concentration (1 ug/mL). The sensitivity of the assay based on absolute on-column injection of CdA was < 1 ng at which the signal to noise ratio at the CdA peak was about 10 for a 1 ng injection (Figure 2). The LLQ was <0.05 ug/mL using 50 uL of plasma sample with a CV of 15%. The intra-assay variations at 0.1 and 1 ug/mL determined from 5 sample batches (n= 4 in each batch) over a 3-month period were 7% and 5% respectively; and inter-assay variations were 17% and 3%, respectively (Table 1). There was no interference from other nucleosides such as fludarabine, 5-fluorouracil, N6-cyclohexyl-adenosine and cytosine-1-β-D-arabinofuranoside. The retention times of a list of therapeutic agents tested for interference are summarized in Table 2. The maximum plasma concentrations of CdA observed were 0.77 ± 0.04 ug/mL at the first sampling time (0.3 h), and declined rapidly to negligible concentrations after 4 hours (< 0.05 ug/mL). There were considerable differences in the plasma concentrations of CdA between rats. The inter-individual differences at the first sample time and at 4 hours were 46% and 65% respectively. No interference was encountered from endogenous materials in the plasma. The mean plasma concentration-time profile of CdA in rats (n = 6) and the pharmacokinetic parameters following a single 2 mg/kg dose administered subcutaneously are shown in Figure 3.

DISCUSSION

There have been only a few HPLC methods published for CdA in plasma to-date (8 , 9). The HPLC method described by Liliemark and co-workers (9) utilized a liquid-liquid extraction to isolate CdA from plasma using ethyl acetate, followed by separation from plasma endogenous substances using reversed phase HPLC and UV
Figure 2: HPLC chromatograms of a standard solution of cladribine, fludarabine and the internal standard AZT (5 ng each). Bottom: Typical standard curve of cladribine in plasma; each point represents mean ± SD of 4 replicates.

Table 1: Intra- and inter-assay variations of the HPLC assay of cladribine in plasma

<table>
<thead>
<tr>
<th>Period / Concentrations</th>
<th>1 ug/mL</th>
<th>0.1 ug/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>%CV</td>
</tr>
<tr>
<td>Batch No 1</td>
<td>1.05 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>Batch No 2</td>
<td>0.99 ± 0.01</td>
<td>3</td>
</tr>
<tr>
<td>Batch No 3</td>
<td>1.01 ± 0.01</td>
<td>2</td>
</tr>
<tr>
<td>Batch No 4</td>
<td>1.06 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>Batch No 5</td>
<td>1.01 ± 0.03</td>
<td>7</td>
</tr>
<tr>
<td>Mean Intra-assay</td>
<td>1.03 ± 0.02</td>
<td>6</td>
</tr>
<tr>
<td>Inter-assay Variation</td>
<td>3%</td>
<td>7</td>
</tr>
</tbody>
</table>

Each value represents mean ± SEM of 4 replicates.

detection. Using 1 mL of plasma samples, the of CdA up to 24 hours after the administration of 0.14 mg/kg administered by a 2 h infusion. The method described by Albertioni et al. employed a retentive SPE technique with C8 columns, followed by reversed phase HPLC separation and UV detection. The recovery of CdA was over 90% and that using 1 mL of plasma sample, the assay had a limit of detection of less than 1 ng/mL (8). The HPLC method described in this paper also used retentive SPE technique coupled with reversed phase HPLC separation and UV detector; however, the SPE columns employed were C18 and that the solvent for desorption was a 10% methanol in ethyl acetate.
Table 2: Retention times of drugs tested for interferences

<table>
<thead>
<tr>
<th>Drug tested</th>
<th>Retention time (min)</th>
<th>Drug tested</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N6-Cyclohexyl-adenosine</td>
<td>&gt; 100 min</td>
<td>Irinotecan</td>
<td>&gt; 60 min</td>
</tr>
<tr>
<td>Cytosine-1-β-D-arabinofuranoside</td>
<td>4 min</td>
<td>Camptothecin</td>
<td>&gt; 60 min</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>7 min</td>
<td>Dipyridamole</td>
<td>&gt; 60 min</td>
</tr>
<tr>
<td>2-Chloroadenosine</td>
<td>13 min</td>
<td>Diltiazem</td>
<td>&gt; 60 min</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>4 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3: Mean plasma concentrations of cladribine in rats following a single 2 mg/kg subcutaneous injection. Each point represents mean ± SEM of 6 rats.

which also provided over 90% recoveries for CdA. The current assay required only 50 µL plasma samples and has a LLQ of less than 0.05 µg/mL (50 ng/mL), and a sensitivity of less than 1 ng assessed by on column injection. In addition, we have found that the HPLC method as described in this paper is very robust, such that different laboratory personnel can repeat the assay with very similar results, as judged by the small inter-assay variability (< 20% over 3 months). We have noted a greater assay variation at lower plasma concentrations than at higher concentrations (Table 1), which is quite typical for SPE. It could be a result of adsorption of CdA or the internal standard AZT on to the SPE column, which was more variable at the lower concentration. Removing one of the 5 values (e.g. batch No 4) reduces the inter-assay variation to 11%. In addition to these advantages, the current method employed a 250 x 2 mm ID minibore column, which minimizes mobile phase consumption, and hence the cost per analysis could be reduced considerably. Furthermore, none of the compounds we have tested interfered with the analysis (Table 1). These include other nucleoside and non-nucleoside anti-cancer and cardiovascular agents, which may enhance the anti-cancer effects of nucleosides anti-cancer agents, and potentially be taken together with CdA in clinical situations.

Using the HPLC assay described in this paper, we were able to measure plasma concentrations of CdA for pharmacokinetic study in rats following a single 2 mg/kg subcutaneous injection (Figure 3). To our knowledge, this is the first report of a complete plasma concentration-time profile of CdA in rats following a single subcutaneous injection. The method can be adopted readily for clinical pharmacokinetic studies by increasing the plasma sample size. Typically, steady-state plasma concentrations of cladribine are in the range of 5 – 10 ng/mL following continuous infusion of 0.1
mg/kg/day (10). The current HPLC uses 50 uL of plasma sample and has a LLQ of about 50 ng/mL. It should be able to measure lower than 5 ng/mL if the plasma sample size is 1 mL or larger. It can also be used for therapeutic monitoring, as it would not be interfered by a wide range of therapeutic agents as tested. In summary, the HPLC assay as described have adequate sensitivity and specificity to determine CdA concentrations in plasma for pharmacokinetics studies in rats, and should be readily adaptable also for clinical pharmacokinetic studies and therapeutic monitoring.

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REFERENCES