Solid-Phase Extraction Combined with Radioimmunoassay for Measurement of Zalcitabine (2',3'-Dideoxyctydidine) in Plasma and Serum

William L. Roberts, Timothy J. Buckley, Petrie M. Rainey, and Peter I. Jatlow

Of the antiviral agents that are currently in clinical use in the US for therapy for human immunodeficiency virus infections, zalcitabine (ddC) is the most potent and is effective at the lowest plasma concentrations. The two reported procedures for measuring these low concentrations involve a chromatographic technique coupled with mass spectrometry. We have developed a procedure combining solid-phase extraction with a strong cation-exchange resin and commercially available RIA reagents for the quantification of ddC in plasma or serum. The method demonstrates good linearity, specificity, and precision, with overall CVs of <10% from 2–20 μg/L and 17% at 0.8 μg/L (the lower limit of quantitation). No significant cross-reactivity with nucleoside analogs other than ddC analogs was noted. The major advantages of this assay are its efficiency and relative simplicity, which should facilitate its performance in many laboratories.

Indexing Terms: AIDS/drug assay/monitoring therapy/nucleoside analogs

Several 2',3'-dideoxynucleosides and related compounds are under investigation for therapy of human immunodeficiency virus (HIV) infection, the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (1–3). Among these compounds, only AZT (3'-azido-3'-deoxystymidin; zidovudine), ddI (2',3'-dideoxyinosine; didanosine), and ddC (2',3'-dideoxyctydidine; zalcitabine) have been approved for clinical use in the US. A variety of clinical trials with these agents are in progress. Zalcitabine is the most potent of the three drugs in cell culture (4–6), and is effective clinically at the lowest plasma concentrations. Peak plasma concentrations achieved 1–2 h after administration of a 0.5-mg tablet were ~7.5 μg/L (7), and, on the basis of a plasma half-life of ~2 h, trough concentrations after the currently recommended dose of 0.75 mg every 8 h should be <1 μg/L. These low concentrations make studies requiring plasma concentration measurements difficult. Conventional analytical methods such as HPLC that have proved useful for AZT and ddI analysis lack the requisite sensitivity. A gas chromatography–mass spectrometry method with a detection limit of 2 μg/L has been described (8) but requires multiple derivatization steps and negative chemical ionization. Recently, an HPLC–thermospray mass spectrometry method has been described with a calibration range down to 0.25 μg/L (9). This method requires sophisticated, expensive instrumentation that is not available in most clinical laboratories. Reagents for an RIA method for ddC are commercially available but the stated limit of quantitation is 3 μg/L, a concentration too high to be useful for pharmacokinetic studies with the currently recommended doses of 0.375 or 0.75 mg administered every 8 h. We report here the development and validation of an assay for ddC that couples solid-phase extraction (SPE) with RIA. This assay has a detection limit of 0.3 μg/L, a limit of quantitation of 0.8 μg/L, and can be performed with readily available instrumentation. It should permit the assessment of patients' compliance and pharmacokinetic characteristics. It may also facilitate the establishment of a therapeutic range, although the clinical significance of extracellular concentrations of unphosphorylated antiretroviral nucleoside analogs is unclear.

Materials and Methods

Materials

Rabbit antiserum to ddC, rabbit IgG immunoprecipitation reagent, [3H]ddC for RIA, fluorescence polarization immunoassay (FPIA) dilution buffer, 2',3'-didehydro-3'-deoxystymidin (d4T), and unlabeled ddC were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]ddC (5 kCi/mol), [3H]AZT (14 kCi/mol), and [3H]ddI (31 kCi/mol) for recovery studies were from Moravek Biochemicals (Brea, CA). We obtained analogs of ddC from Y. C. Cheng, Yale University. Bond-Elut 100-mg and 500-mg strong cation-exchange (SCX) columns were purchased from Varian Associates (Harbor City, CA). Ecoscint scintillation cocktail was from National Diagnostics (Manville, NJ). All reagents and solvents were at least of reagent grade. Serum specimens were collected into Vacutainer Tubes (Becton Dickinson, Franklin Lakes, NJ) without anticoagulant; plasma specimens were collected in Vacutainer Tubes with lithium heparin. All specimens were centrifuged at 1000g for 5 min. A pool of citrated human plasma was prepared by combining six units of outdated fresh frozen plasma obtained from the Connecticut Red Cross. Calibrators containing 0, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25 μg/L ddC were prepared with the pooled human plasma.

Received August 19, 1993; accepted September 29, 1993.

1 Address correspondence to this author. Fax 203-785-7340.
2 Nonstandard abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; AZT, 3'-azido-3'- deoxystymidin (zidovudine); ddI, 2',3'-dideoxyinosine (didanosine); ddC, 2',3'-dideoxyctydidine (zalcitabine); SPE, solid-phase extraction; FPIA, fluorescence polarization immunoassay; SCX, strong cation-exchange; d4C, 2',3'-dideoxy-3'-thiactydine; OddC, 1-[2-(hydroxy- methyl)-1,3-dioxolan-2-y]cytosine; and ddT, 2',3'-didehydro-3'-deoxystymidin.

3 A proprietary buffer for FPIA and RIA.
Apparatus

We used a 12-port homemade vacuum manifold for SPE. Any of several commercial models should be equally usable. Scintillation counting was performed on an LS-7500 scintillation counter (Beckman Instruments, Palo Alto, CA).

Procedures

Solid-phase extraction. SCX columns were prewashed with 3 mL of methanol followed by 3 mL of water. We diluted aliquots (2 mL) of calibrators, controls, and samples with 2 mL of 2 mol/L phosphoric acid and applied them to the columns on a vacuum manifold. After sample application, the columns were washed with 4 mL of water and 4 mL of methanol and eluted with 4 mL of 20 mL/L concentrated ammonium hydroxide solution in methanol into 13 x 100 mm glass culture tubes. Eluates were dried under a stream of compressed air at room temperature in a fume hood and subsequently dissolved with vortex-mixing in 250 μL of FPFA buffer. We performed recovery studies for the column extraction procedure by supplementing 2-mL plasma samples with [3H]ddC, [3H]AZT, and [3H]ddl (0.02 μCi per replicate).

RIA. Each binding reaction was carried out in a 12 x 75 mm glass culture tube by combining 100 μL of redissolved extract in FPFA buffer, 100 μL of [3H]ddC, and 100 μL of ddC antisera. Standards, controls, and samples were analyzed in duplicate. We determined nonspecific binding in the absence of ddC antisera. After gentle mixing followed by a 1-h room-temperature incubation, we added 1 mL of rabbit IgG immunoprecipitation reagent to each tube with gentle mixing and centrifuged the samples at 2000 g for 15 min at 4°C. The supernatants were decanted and the precipitates dissolved in 600 μL of 100 mmol/L HCl. The total-count tubes contained 500 μL of 100 mmol/L HCl added to 100 μL of [3H]ddC. A 500-μL aliquot from each tube was counted in 5 mL of scintillation fluid for 5 min. Assay data were analyzed by RiaSmart RIA/QC software from Packard Instrument Co. (Meriden, CT), using a spline fit.

RIA validation. We assessed precision by analyzing four concentrations of ddC (0.8, 2, 10, 20 μg/L) in human plasma in quintuplicate on 5 separate days. To assess the linearity of the assay, we pooled the plasma obtained from three patients who were receiving ddC and serially diluted it with drug-free plasma. In addition, 1- and 2-mL aliquots of three ddC quality controls (2, 10, and 20 μg/L) were assayed concurrently and the measured concentrations were corrected for the aliquot volume.

We evaluated the specificity of the rabbit antisera to ddC by measuring the cross-reactivity of anti viral and antimitabolite nucleoside analogs, including AZT, ddI, cytosine arabinoside, 5-fluorouracil, acyclovir, ganciclovir, and the ddC analogs (+)- and (−)-2',3'-dideoxy-3'-thiacytidine (SddC) and (−)-1,2-(hydroxymethyl)-1,3-dioxolan-5-yl)cystosine (OddC) pre-pared in aqueous solution. We also prepared ddC standards in pooled serum from patients receiving AZT. The concentration of AZT in the serum pools was quantified with a ZDV-Trac RIA (Incstar Corp., Stillwater, MN). The analytical recovery of unlabeled ddC from control, lipemic, icteric, and hemolyzed serum specimens was compared with that from plasma.

We determined a pharmacokinetic profile by administering a 0.75-mg dose of ddC to a 95-kg male volunteer and obtaining plasma samples at 0, 0.5, 1.0, 1.5, 2.0, 3.5, 5.0, 6.5, and 8.0 h thereafter. Pharmacokinetic characteristics were estimated by using standard model-independent techniques (10). Protocols involving human subjects were approved by the Human Investigation Committee of the Yale University School of Medicine or by the institutional review board of the specimen-collection site.

Results

We compared the extraction efficiency and reproducibility of 100- and 500-mg SCX columns by using [3H]ddC. Recoveries from the larger columns averaged 89% (n = 3, SD = 1.7%) but were <50% from the smaller ones when 2-mL aliquots of serum or plasma were extracted. Therefore, the larger columns were used in all subsequent experiments. The recoveries of [3H]ddC from 2-mL aliquots of both serum and plasma were identical, and the presence or absence of 20 ng of unlabeled ddC had no effect. The recoveries of both [3H]AZT and [3H]ddl from plasma by SCX extraction were 5%.

The limit of detection of SPE coupled with RIA, calculated by the RiaSmart software and defined as 1 SD of duplicate determinations of the zero calibrator, was 0.1 μg/L. A more realistic estimate of the limit of detection is 0.3 μg/L. The intra- and interday precision of the method was determined by using four concentrations of quality controls prepared from outdated pooled human plasma with added ddC. The results are summarized in Table 1. The mean for each concentration was within 10% of the nominal value, and the overall CVs of the 2, 10, and 20 μg/L concentrations were <10%. The limit of quantitation as defined by accuracy within 20% of the nominal value and a CV of ≤20% (11) was empirically determined to be <0.8 μg/L by repetitive assay of 0.8 μg/L control material. This ddC concentration corresponds to a 20% displacement of bound [3H]ddC in the RIA.

The linearity of the assay was assessed by serial dilution of plasma pooled from patients receiving ddC with drug-free plasma. Linear regression of the measured (y) vs expected (x) concentrations of ddC gave the equation y = 1.02x - 0.3 (R² = 0.996; standard error of the estimate = 0.82). To further check linearity, we

| Table 1. Precision of zalcitabine SPE-RIA.  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc, μg/L</td>
<td>Mean ± SE, μg/L</td>
<td>Interday CV, %</td>
<td>Intraday CV, % (range)</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
<td>-------------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>0.8</td>
<td>0.79 ± 0.03</td>
<td>16.6</td>
<td>6.4-19.2</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.79 ± 0.03</td>
<td>7.4</td>
<td>1.7-7.3</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>9.57 ± 0.17</td>
<td>8.6</td>
<td>5.8-7.9</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>19.03 ± 0.33</td>
<td>8.8</td>
<td>2.6-13.8</td>
<td></td>
</tr>
</tbody>
</table>

* Quintuplicate determinations on each of 5 separate days.
assayed 1- and 2-mL aliquots of the three highest controls in parallel. The apparent concentrations obtained with the 1-mL aliquots were within 10% of half the value obtained with the 2-mL aliquots.

We assessed the specificity of the RIA reagents in two ways. First, the cross-reactivity of several nucleoside and pyrimidine analogs in aqueous solution was determined without SCX extraction (Table 2). Significant cross-reactivity was detected only for ddC analogs and not for any other compounds tested. Because ddC is often used in combination with AZT, a second test of cross-reactivity was performed to rule out interference by AZT metabolites. Serum pools from patients receiving AZT therapy at concentrations of 125 and 160 μg/L were supplemented with ddC to produce the concentrations present in the calibrators and asayed for ddC. Immune identity with the calibrators was demonstrated for each. The results from one experiment are shown in Fig. 1.

We assessed the reactivity of blank serum by analysis of plasma from eight patients not receiving ddC. No sample had detectable ddC. The effect of three common interferences was also examined. The presence of lipemia (triglyceride 2 g/L), hemolysis (hemoglobin 2.41 g/L), and hyperbilirubinemia (total bilirubin 110 mg/L) had no effect on the recovery of ddC from serum.

A pharmacokinetic profile of ddC concentrations measured when a healthy male volunteer was given a standard 0.75-mg dose of ddC is shown in Fig. 2. The peak concentration observed 1.5 h after administration was 5.3 μg/L and the apparent elimination half-life was 2.5 h. The concentration 8 h after administration of a single dose, which would correspond to a trough concentration with a standard 8-h dosing interval, was 1.0 μg/L. The oral volume of distribution (which corresponds to the volume of distribution divided by the oral bioavailability) was 1.27 L/kg, and the oral clearance (clearance divided by the oral bioavailability) was 5.5 mL/min per kg.

**Table 2. Cross-reactivity of selected compounds in the ddC RIA.**

| Compound     | Concentration, μg/L | Cross-reactivity, %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-SddC</td>
<td>69</td>
<td>160</td>
</tr>
<tr>
<td>(-)-SddC</td>
<td>229</td>
<td>9.0</td>
</tr>
<tr>
<td>(+)-OddC</td>
<td>213</td>
<td>4.6</td>
</tr>
<tr>
<td>(-)-OddC</td>
<td>2130</td>
<td>0.6</td>
</tr>
<tr>
<td>d4T</td>
<td>18 000</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>ddI</td>
<td>20 000</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>AZT</td>
<td>30 000</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ara-C</td>
<td>100 000</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>100 000</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>100 000</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>100 000</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Cross-reactivity of each compound was determined on a weight basis by RIA of duplicate 100-μL aliquots of aqueous solutions prepared at the indicated concentrations. A calibration curve was constructed by extracting 2-mL aliquots of ddC in plasma, resuspending the extracts in 250 μL, and assaying duplicate 100-μL aliquots of each calibrator. Assuming an 89% recovery of each calibrator after extraction, the actual concentration of ddC of each calibrator was used in the spline fit of the calibration curve.

Ara-C, cytosine arabinoside.

**Discussion**

Commercially available reagents used to measure ddC by RIA include rabbit antiserum to a ddC–bovine serum albumin conjugate, rabbit IgG immunoprecipitation reagent, and [3H]ddC. These reagents are not in a kit, but a suggested protocol for their use is provided by the manufacturer. This protocol has not yet been formally validated. The stated limit of detection is 3 μg/L,
which is inadequate for measurement beyond 3 h after the currently recommended dosage, and is thus not suitable for pharmacokinetic or trough concentration monitoring. To improve the sensitivity of this assay, we used a SPE procedure to concentrate the ddC. Cytidine has an amino group with a pK of 4.15 (12). After acidification with phosphoric acid, cytidine and its analogs should be readily extracted from serum or plasma by an SCX resin. After washing with water and methanol at neutral pH, ddC can be eluted from the resin by methanolic ammonium hydroxide, which deprotonates the amino group.

The coupling of SPE with RIA provided several benefits. First, SPE concentrated ddC by about sevenfold, thereby decreasing the lower limit of quantitation by a similar factor. The Sigma protocol states that as little as 0.3 ng of ddC may be detected by the RIA. Because the assay includes a 100-μL aliquot of plasma, this corresponds to a concentration of 3 μg/L. No statements regarding the precision of this assay are provided. The method described here has a limit of quantitation of <0.8 μg/L, thereby achieving a fourfold increase in usable sensitivity. The trough (8-h) concentration measured in a healthy volunteer was measurable with our assay, and the pharmacokinetic measurements were compatible with the range of values obtained by gas chromatography–mass spectrometry in patients with AIDS or AIDS-related complex (7). The ability of this assay to measure ddC concentrations over the entire dosing interval not only facilitates pharmacokinetic studies but should allow measurement of pharmacokinetic characteristics other than peak concentrations (e.g., trough concentrations or area under the time-vs-concentration curve) that may correlate better with efficacy and toxicity.

Second, SPE may increase selectivity. This was demonstrated by the low recoveries (5%) of AZT and ddI. Although these substances were not found to cross-react to a significant extent in the RIA, other unidentified substances that have the potential to cross-react with ddC antiserum may be removed by the relatively selective extraction achieved by SCX. Third, extraction with an SCX resin should diminish the concentration of anionic substances such as phospholipids and fatty acids, which have been reported to interfere with a variety of immunoassays (13 and references therein).

Several nucleosides that contain cytosine and uracil moieties were previously known to cross-react with ddC antiserum. Whereas the cross-reactivity of cytidine and 2'-deoxycytidine were 0.01% and 0.12%, respectively, 2',3'-dideoxyuridine, which contains dideoxyribose linked to a pyrimidine base, exhibited 4.1% cross-reactivity on a molar basis (Sigma). However, we found that the cross-reactivity of ddI, which contains dideoxyribose linked to a purine base, was <0.02% and that of ddT, which contains didehydroribose linked to a pyrimidine base, was <0.02% (Table 2).

Significant cross-reactivity was detected with the ddC analogs (+)- and (−)-SddC (14, 15) and (+)- and (−)-OddC (16), which are candidate antiviral agents for treatment of HIV and hepatitis B infections. This suggests that our assay could be adapted to measure these ddC analogs, some of which attain significantly higher plasma concentrations than ddC (17). The carbon at the 3' position of the dideoxyribose has been replaced by a sulfur atom in the SddC analogs. Since the (+) isomer is in the same conformation as ddC, it is not surprising that substantial cross-reactivity was detected with this compound. The (−) isomer, which is under clinical investigation for the treatment of HIV infection, shows 9% cross-reactivity in the ddC RIA. The analogs of these compounds, in which the sulfur atom has been replaced with oxygen, show less cross-reactivity. The analog (+)-OddC, which has the same conformation as ddC, shows 4.6% cross-reactivity; (−)-OddC shows only 0.6%. It is unlikely that the agents mentioned above having substantial cross-reactivity would be administered concurrently with ddC. Cytosine arabinoside, which contains a cytosine moiety but has arabinose in place of dideoxyribose, showed no cross-reactivity with the antibody. The above data suggest that the antibodies to ddC recognize epitopes that include portions of both the cytosine and dideoxyribose moieties. Several other chemotherapeutic agents frequently administered to patients with AIDS showed no cross-reactivity in the assay.

In summary, we have developed an SPE-RIA for ddC that shows excellent linearity and precision for plasma concentrations of the drug that are observed clinically. The commercially available antisera shows high specificity for ddC and its analogs. The major advantage of this assay over other reported assays is that it can be readily performed in many laboratories.

This work was supported in part by National Institutes of Health grant AI23766 (P.I.J. and P.M.R.) and by a Merck/American Federation for Clinical Research Foundation postdoctoral fellowship (W.L.R.). The nucleoside analogs (+)- and (−)-SddC and (+)- and (−)-OddC were graciously provided by Y. C. Cheng. We thank R. Heimer for constructive discussions.

References


