Zalcitabine Population Pharmacokinetics: Application of Radioimmunoassay

JOHN M. ADAMS,1,2,3 MARK J. SHELTON,1,2,3 ROSS G. HEWITT,3,4,5 MARY D. DEREMER,1 ROBIN DIFRANCESCO,1 THADDEUS H. GRASELA,2 and GENE D. MORSE1,2,3,4,5

Laboratory for Antiviral Research* and Departments of Pharmacy Practice and Medicine, State University of New York at Buffalo, and Antiviral Clinical Pharmacology Unit, Immunodeficiency Clinic, Erie County Medical Center, Buffalo, New York, and AIDS Clinical Trials Unit, University of Rochester School of Medicine, Rochester, New York

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Zalcitabine population pharmacokinetics were evaluated in 44 human immunodeficiency virus-infected patients (39 males and 5 females) in our immunodeficiency clinic. Eighty-one blood samples were collected during routine clinic visits for the measurement of plasma zalcitabine concentrations by radioimmunoassay (1.84 ± 1.24 samples/patient; range, 1 to 6 samples/patient). These data, along with dosing information (age 38.6 ± 7.13 years, sex, weight (79.1 ± 15.0 kg), and estimated creatinine clearance (89.1 ± 21.5 ml/min), were entered into NONMEM to obtain population estimates for zalcitabine pharmacokinetic parameters (4). The standard curve of the radioimmunoassay ranged from 0.5 to 50.0 ng/ml. The observed concentrations of zalcitabine in plasma ranged from 2.01 to 8.57 ng/ml following the administration of doses of either 0.375 or 0.75 mg. A one-compartment model best fit the data. The addition of patient covariates did not improve the basic fit of the model to the data. Oral clearance was determined to be 14.8 liters/h (0.19 liter/h/kg; coefficient of variation [CV] = 23.8%), while the volume of distribution was estimated to be 87.6 liters (1.18 liters/kg; CV = 54.0%). We were also able to obtain individual estimates of oral clearance (range, 8.05 to 19.8 liters/h), volume of distribution (range, 49.2 to 161 liters; 0.43 to 1.92 liters/kg) of zalcitabine in these patients with the POSTHOC option in NONMEM. Our value for oral clearance agrees well with other estimates of oral clearance from traditional pharmacokinetic studies of zalcitabine and suggests that population methods may be a reasonable alternative to these traditional approaches for obtaining information on the disposition of zalcitabine.

Zalcitabine has been extensively evaluated as monotherapy and in combination regimens for patients with human immunodeficiency virus (HIV) infection (11, 15, 30). Early trials of zalcitabine monotherapy noted an unacceptable incidence of peripheral neuropathy (5, 10, 22–24, 25, 31, 32, 38). As a result, subsequent trials were designed to evaluate lower doses (12, 13, 22, 25, 31). Zalcitabine is usually prescribed at dosages of 0.375 or 0.75 mg every 8 h. However, certain aspects of zalcitabine therapy such as optimal dosage reductions or dosing interval adjustments in patients with decreased renal function or receiving dialysis remain to be clarified.

One obstacle to the further refinement of zalcitabine dosing has been the lack of an assay which is sensitive enough to reliably quantitate concentrations in plasma after the administration of currently prescribed doses. Phase I pharmacokinetic studies used a high-performance liquid chromatography assay; however, this method is not sensitive enough for the study of current doses (17–21). Other techniques such as combined gas or liquid chromatographic-mass spectrometric assays have been reported (28); however, their application to clinical studies of current zalcitabine doses has not been evaluated.

The reagents for a zalcitabine radioimmunoassay have been available for quite awhile, and a prior report described the enhanced sensitivity of this assay method (7). However, the description of this method also indicated the need for an initial solid-phase sample preparation step, which requires 0.5 ml per sample. We have also validated this radioimmunoassay method, but without an additional sample preparation step and with a requirement for 200 µl per sample. This report summarizes the assay validation and describes its use in a population pharmacokinetic analysis of zalcitabine.

MATERIALS AND METHODS

Chemicals and reagents. Zalcitabine and the primary and secondary antibodies were purchased from Sigma Chemical Company Inc. (St. Louis, Mo.) [2,3]. Zalcitabine was purchased from Moravek Biochemicals Inc. (LaBrea, Calif.). The following reagent-grade chemicals were purchased from Fisher Scientific (Rochester, N.Y.): concentrated hydrochloric acid, sodium azide, and monobasic and dibasic sodium phosphate. Ecoscent scintillation fluid was obtained from National Diagnostics (Manville, N.J.). Knox brand gelatin (Knox Gelatine, Inc. Englewood Cliffs, N.J.) was used for the assay buffer. Assay buffer contained 0.01% gelatin and 0.02% sodium azide in 0.02 M sodium phosphate (pH 7.4). Dilution buffer was obtained from zidovudine radioimmunoassay kits (INCStar, Stillwater, Minn.).

Working dilutions of antibody and tracer were prepared as follows: antizalcitabine rabbit antisem, 0.05 to 0.10 mg/ml in assay buffer; [3H]zalcitabine, 0.002 nCi/ml in assay buffer. Calibration standards were prepared as various dilutions of a 0.1-mg/ml stock of zalcitabine in assay buffer, diluting with dilution buffer to yield concentrations of 0.6, 1.0, 1.6, 2.2, 3.0, 4.0, 10, 16, 24, 33.3, and 50 ng/ml. The standards were stored at −20°C in screw-top cryotubes in aliquots with 1- and 4-ml volumes. Quality control samples were diluted from a separately prepared 0.1-mg/ml stock in assay buffer to attain 1.3, 2.7, 12, and 30 ng/ml and were aliquoted and stored in the same manner as the calibration standards.

Radioimmunoassay. All reagents were equilibrated to room temperature before use. One hundred microliters of each standard, control, and plasma unknown was pipetted into borosilicate glass tubes (12 by 75 mm) in duplicate. To ensure the best curve fit, the calibration standards at the lowest three dilutions and the first two quality controls were assayed in quadruplicate. No sample preparation step was used. Two hundred microliters of dilution buffer was added to nonspecific binding tubes and 100 µl was added to reference tubes. One hundred microliters of diluted tracer was added to every tube. One hundred microliters of the antizalcitabine was added to each tube except the nonspecific binding and total counts tubes. All tubes were vortexed briefly and were incubated at room temperature for 1 h. Secondary antibody, goat antirabbit antisem (250 µl), was added to all tubes except the total counts tube. The tubes
were vortexed briefly, incubated at 4°C for 30 min, and then centrifuged at 2,200 × g for 45 to 60 min and the supernatant was decanted. The pellet was dissolved in 200 μl of 0.1 N HCl and transferred to a scintillation vial containing 5 ml of scintillation fluid. The vials were vortexed vigorously and counts were measured for 20 min on a Wallac model 1409 liquid scintillation counter (Wallac, Gaithersburg, Md.).

Quantitation. The disintegrations per minute for the calibration standards were fitted to a smooth spline function by using RIACalc software, version 2.65 (Wallac). Calibration standards within the quantitation range (2 to 50 ng/ml) were deleted if they were not within ±20% of the nominal value and the coefficient of variation (CV) of the duplicates was not ≤20%. The curve fit was considered acceptable if five or more points remained. The concentrations in the quality control and plasma unknown samples were then derived from this curve. Quality controls were considered acceptable if they had concentrations within ±20% of the nominal values and had a ≤20% CV between duplicates.

Method validation. The fitted calibration standard values and quality control values were used to determine assay variation. Statistics for the mean and standard deviation of fitted calibration standard values and quality controls were calculated with Lotus 1-2-3, version 4.0, software. The percent CV was calculated as [(mean − true value)/true value] × 100. Blank plasma samples from 14 individuals not receiving zalcitabine were analyzed to determine if there were false-positive measurements. Four of the samples were repeated with four or six replicates on 3 other days to test variations in the background from assay to assay.

The specificity of the assay was also evaluated by testing for interference from other nucleoside analogs including zidovudine, didanosine, and stavudine. Interference by anticonvulstant medications was also tested by assaying samples with clinically relevant concentrations of doxepin, fluoxetine, ranitidine, pyrimethamine, pyrazinamide, ibuprofen, diphenhydramine, penicillin G, and sulfamethoxazole.

Data on the stability of plasma samples containing zalcitabine, which were either refrigerated or frozen, were obtained by assaying high- and low-quality control standards at 3.2 ng/ml and above. The samples were stored at −20°C until its contents were assayed. Patient clinic charts were reviewed to verify zalcitabine dosing histories and to obtain pertinent demographic information.

Prior to assay, all samples were heat inactivated in a water bath for 30 min at 56°C. Plasma zalcitabine concentrations were determined by the radioimmunoassay method described above. The concentrations of zalcitabine in plasma and dosing history information were input into a Lotus database and were later converted into a NONMEM-ready input file. Various models for determining the population pharmacokinetic parameters of zalcitabine were evaluated with the nonlinear regression program NONMEM, version IV, level 1.0. Potential fixed effects on oral clearance (total body weight, age, gender, calculated creatinine clearance, administration of zalcitabine and zidovudine) and volume of distribution (total body weight) were considered in the development of the model. Model discrimination criteria included the minimization of the objective function value, precision of parameter estimates, and the magnitude of residual variability. Finally, individual estimates of zalcitabine oral clearance and volume of distribution were obtained for each patient by using the POSTHOC option within NONMEM (3, 29).

RESULTS

Radioimmunoassay. Table 1 shows the calibration standard and quality control values generated during the measurement of zalcitabine concentrations in specimens. The variation between duplicates at concentrations below 2 ng/ml often exceeded 20%; therefore, measurements were done in quadruplicate. Calibration standards of 0.5, 0.8, and 1.5 ng/ml were omitted if the fitted value differed by more than 25% from the target value; other calibration standards were omitted if the fitted value differed from the target value by more than 15%. More calibration standards were omitted at the lower end of the concentration range, where the variability of fit was higher. The 1.0-ng/ml quality control standard reflected this variation, displaying a CV of 27% and an accuracy of −12%. Calibration standards at 3.2 ng/ml and above had consistently better fits with less variation. The 10-ng/ml quality control standard was also much less variable, exhibiting 9.5% variation and ±4% accuracy.

The results for blank plasma specimens from 14 individuals ranged from 0 to 0.9 ng/ml; therefore, the limit of quantitation was established as 2 ng/ml. Of the 20 compounds tested for cross-reactivity, only ranitidine and didanosine exhibited measurable cross-reactivity at 0.10 mg/ml. Results for stability testing showed that the controls containing both high and low concentrations of zalcitabine showed no statistical differences either for storage effect or for freeze-thaw cycles.

The recovery of zalcitabine from plasma samples spiked with
the drug at 1.0 to 10 ng/ml is illustrated in Fig. 1. The samples with greater than 2.0 ng/ml were assayed with and without dilution. For concentrations above 2.0 ng/ml, the overall mean values indicated that the assay is accurate within ±30% of the target value. Percent error did not improve with dilution of the sample.

**Patients.** A total of 44 patients (39 males and 5 females) were enrolled, with 81 samples available for the analysis (1.84 ± 1.24 samples/patient; range, 1 to 6 samples/patient). The demographic characteristics of these patients are summarized in Table 2.

**Pharmacokinetics.** The observed concentrations of zalcitabine in plasma ranged from 2.01 to 8.57 ng/ml following the administration of a dose of either 0.375 or 0.75 mg (Fig. 2). A one-compartment model with first-order elimination best fit the data. The complete pharmacostatistical model is presented in Table 3. None of the evaluated fixed effects on oral clearance (total body weight, age, gender, calculated creatinine clearance, administration of zalcitabine with food, and concomitant administration of zalcitabine and zidovudine) or volume of distribution (total body weight) improved the fit of the basic model to the data. The population estimate for oral clearance was determined to be 14.8 liters/h (0.19 liter/h/kg; 95% confidence interval, 1.17 to 1.30 liters/h/kg), which agrees well with a previous estimate reported from traditional pharmacokinetic studies of zalcitabine (36). The CV associated with this estimate of oral clearance was 23.8%. The volume of distribution was estimated to be 87.6 liters (1.18 liters/kg; 95% confidence interval, 1.07 to 1.30 liters/kg), with a CV of 54.0%. The absorption rate constant could not be modeled because of the paucity of blood samples collected early in a dosing interval. Residual variability was estimated to be 20.6% with a proportional error model. Finally, we were able to obtain individual estimates of oral clearance (range, 8.05 to 19.8 liters/h; 0.11 to 0.30 liters/h/kg) and volume of distribution (range, 49.2 to 161 liter; 0.43 to 1.92 liters/kg) of zalcitabine in these patients with the POSTHOC option in NONMEM.

**DISCUSSION**

The pharmacokinetics of zalcitabine have been studied to a limited extent, primarily due to a lack of availability of an assay which is sensitive enough to measure concentrations in the plasma of patients receiving the currently recommended doses (37). The dose-limiting toxicities of zalcitabine, primarily peripheral neuropathy, occur in an unpredictable manner and at different times after the initiation of therapy (5, 6, 24, 30). While zalcitabine is one of the more potent of the approved

**TABLE 2. Patient demographics**

<table>
<thead>
<tr>
<th>Demographic characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (no. [%] of patients)</td>
<td></td>
</tr>
<tr>
<td>Male ..........................</td>
<td>39 (89)</td>
</tr>
<tr>
<td>Female ................................</td>
<td>5 (11)</td>
</tr>
<tr>
<td>Race (no. [%] of patients)</td>
<td></td>
</tr>
<tr>
<td>Caucasian ........................</td>
<td>32 (73)</td>
</tr>
<tr>
<td>African American ................</td>
<td>4 (9)</td>
</tr>
<tr>
<td>Caucasian ........................</td>
<td>7 (16)</td>
</tr>
<tr>
<td>American Indian ..................</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Risk factor (no. [%] of patients)</td>
<td></td>
</tr>
<tr>
<td>Homosexual activity ............</td>
<td>28 (64)</td>
</tr>
<tr>
<td>Intravenous drug use ............</td>
<td>7 (16)</td>
</tr>
<tr>
<td>Other/unknown ..........................</td>
<td>9 (20)</td>
</tr>
<tr>
<td>Clinical situation (mean ± SD [range])</td>
<td></td>
</tr>
<tr>
<td>Age (yr) ..........................</td>
<td>38.6 ± 7.13 (27–56)</td>
</tr>
<tr>
<td>Wt (kg) ...........................</td>
<td>79.1 ± 15.0 (46.5–123)</td>
</tr>
<tr>
<td>CL$_{cr}$ (ml/min) ................</td>
<td>89.1 ± 21.5 (53.6–146)</td>
</tr>
</tbody>
</table>

* A total of 44 patients were studied. Thirty-nine of the 44 patients were concomitantly receiving zidovudine. CD4 counts ranged from 6 to 540 cells/mm$^3$ (mean, 165 cells/mm$^3$) in a subset of these patients.

**TABLE 3. Pharmacostatistical model and parameter estimates**

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pharmacokinetics</strong></td>
<td>TVCL = $\theta_1^b$</td>
</tr>
<tr>
<td></td>
<td>TVV = $\theta_2^b$</td>
</tr>
<tr>
<td></td>
<td>CL = TVCL $\cdot$ EXP($\eta_1$)$^\gamma$</td>
</tr>
<tr>
<td></td>
<td>V = TVV $\cdot$ EXP($\eta_2$)$^\gamma$</td>
</tr>
<tr>
<td><strong>Error</strong></td>
<td>$y = F + F \cdot \epsilon_i$</td>
</tr>
<tr>
<td><strong>Mean parameter estimates (% CV)</strong></td>
<td>CL/F = 0.19 liter/h/kg (range, 0.11–0.30 liter/h/kg) (23.8)</td>
</tr>
<tr>
<td></td>
<td>V/F = 1.18 liters/h/kg (range, 0.43–1.92 liters/kg) (54.0)</td>
</tr>
<tr>
<td></td>
<td>Residual variability = 20.6%</td>
</tr>
</tbody>
</table>

* Abbreviations b, variables representing parameter values; $\eta_1$, proportional differences from typical values; $\epsilon_i$, difference (proportional) between observed and predicted concentrations; CL, clearance; V, volume of distribution; Y, observed concentration; F, predicted concentration.

* Typical value of clearance divided by predicted concentration or volume of distribution divided by predicted concentration (population estimate).

* Individual estimate of clearance divided by predicted concentration or volume of distribution divided by predicted concentration.

* Proportional error model.
nucleoside analog reverse transcriptase inhibitors, little is known about its intracellular metabolism in patients. As with the measurement of intracellular anabolites for zidovudine and didanosine, the analytical ability to measure the phosphorylated anabolites of zalcitabine in clinical samples has not proceeded rapidly and has been limited to in vitro investigations (1, 2, 8, 9, 26, 33–36). Therefore, the clinical pharmacokinetics of zalcitabine remain the most likely tool for assessing the relationship between concentrations in plasma and surrogate marker responses.

Burger et al. (7) described a false-positive recovery of zalcitabine from blank plasma specimens, measuring up to 3.00 ng/ml, and from urine samples, measuring up to 4.00 ng/ml. Our assay results also found that false detection of zalcitabine was apparent; however, the amounts that we found were less: up to 1.26 ng/ml for plasma. This is likely attributable to the concentration of primary antibody we used. Burger et al. (7) used a dilution threefold higher than that suggested by the manufacturer. Our approach used a sixfold higher dilution of the same product. On the basis of these observations and the variation that we experienced during the measurement of lower zalcitabine concentrations, the lowest concentration reported for our population pharmacokinetics study was 2.0 ng/ml. Any plasma sample with a zalcitabine concentration below this was considered to be indistinguishable from blank plasma.

The availability of the zalcitabine radioimmunoassay also makes the application of population pharmacokinetics a possibility. Zalcitabine is usually dosed every 8 h, and the data presented in Fig. 2 indicate that at the recommended doses, plasma zalcitabine concentrations are measurable by the assay described here. These concentrations in plasma can, in turn, be used to generate population pharmacokinetic parameter estimates and estimates of individual zalcitabine clearance, which can be used to determine individual drug exposures for use in pharmacodynamic studies. Our population estimate of oral clearance was 14.8 liters/h (CV = 23.8%). This value (0.19 liters/h/kg) agrees well with an estimate reported from traditional pharmacokinetic studies and would suggest that random collection of blood samples during routine clinic visits provides a reasonable method for estimating zalcitabine clearance (14, 19, 27, 31). However, efforts to obtain a more even distribution of samples collected at various times after the administration of a dose would assist in more accurately describing population parameters.

Our estimate of volume of distribution (1.18 liters/kg) is larger and more variable than what has been reported in previous pharmacokinetic studies that used the traditional two-stage approach. This could be due in part to the fact that we obtained relatively few blood samples early in a dosing interval. Indeed, we were unable to obtain an estimate for the absorption rate constant for the same reason. Future studies aimed at estimating these population pharmacokinetic parameters should attempt to obtain several blood samples from different patients during this period, perhaps through the controlled administration of a dose of zalcitabine in the clinic followed by blood sampling. Indeed, controlled administration of zalcitabine doses in the clinic would greatly reduce the error associated with patients not accurately remembering when they took their previous dose. This would be particularly important for drugs, like zalcitabine, with a short half-life.

None of the fixed effects that we examined (age, sex, total body weight, calculated creatinine clearance, coadministration of zalcitabine with food, and concomitant zalcitabine and zidovudine therapy) improved the fit of the basic model to the data. This could be at least partially explained by the fact that most of the factors were not well distributed across the range of values. For example, many patients had similar calculated creatinine clearances, despite a range of 54 to 146 ml/min. Similarly, a significant majority of the patients were white males whose risk factor for HIV infection was homosexual activity. Future efforts should be directed toward enrolling patients who can provide more heterogeneity for the analysis of these factors. In addition, the collection of larger numbers of blood samples from each patient would further assist in the estimation of individual pharmacokinetic parameters.

Zalcitabine is primarily renally excreted, and therefore, dosage requirements are most likely needed for HIV-infected patients with reduced renal function. However, current dosing guidelines do not include specific recommendations for adjusting the dose of zalcitabine and suggest that estimates of creatinine clearance be used to guide dosage changes. Furthermore, a recent report has examined the estimates of creatinine clearance obtained with the common nomograms used clinically and compared them to a measured creatinine clearance and found that the calculated estimate overpredicts the measured value for a majority of patients (16). As a result, zalcitabine dosing in patients with renal impairment will be inaccurately predicted and patients may receive excessive doses, possible leading to the development of toxicity.

In summary, the use of chronic zalcitabine therapy is often complicated by the development of peripheral neuropathy. The use of lower doses to avoid neuropathy may in turn reduce the ratio of the concentration in plasma to the 50% inhibitory concentration over each dosing interval. Although unproven, this relationship may be important in clinical outcomes. Since no results from intracellular studies with which zalcitabine dosing can be adjusted are available, the current approach to zalcitabine dosing does not allow for individualization. In addition, patients with reduced renal function may accumulate zalcitabine and may become predisposed to peripheral neuropathy. The application of the zalcitabine radioimmunoassay and population pharmacokinetic methods may allow clinical research to investigate these issues and help to provide a more individualized approach to zalcitabine dosing. Individualized approaches to zalcitabine dosing need to be investigated for their potential clinical utility in maximizing therapeutic outcomes.

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REFERENCES


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