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Didanosine Measurement by Radioimmunoassay

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Didanosine is commonly prescribed as monotherapy or as part of a combination regimen for patients with human immunodeficiency virus infection. The use of lower doses, either as part of a combination regimen or as a result of dose reduction secondary to clinical intolerance, requires that a sensitive assay method be available for either traditional or population-based pharmacokinetic evaluations. We evaluated a radioimmunoassay technique with a standard curve range of 0 to 100 ng/ml in human plasma, urine, and cerebrospinal fluid and assessed its accuracy and precision for use in pharmacokinetic studies.

Didanosine has been approved for the treatment of patients with human immunodeficiency virus infection since 1992. Clinical pharmacokinetic studies which were done as part of the approval process used a high-performance liquid chromatography (HPLC) assay which has a lower limit of sensitivity of 25 ng/ml in plasma and 1.0 μg/ml in urine (11). Didanosine has been shown to have a bioavailability range of from ~30 to 80% among human immunodeficiency virus-infected adults and children (2, 6, 7, 9, 10, 12). The more recent use of didanosine in combination with other antiretroviral agents has led to the evaluation of lower daily doses (3, 5, 14, 15, 18). In addition, many patients require dose reductions because of adverse gastrointestinal effects or other more severe toxicities (i.e., pancreatitis and peripheral neuropathy) (6, 12, 17). Therefore, with many patients taking lower doses of didanosine, the conduct of clinical pharmacokinetic studies will require assay methodologies with sensitivities greater than that of the HPLC technique. This report describes our experience with the measurement of didanosine by a radioimmunoassay.

MATERIALS AND METHODS

Chemicals and reagents. Didanosine, the primary and secondary antibodies, and cell culture-grade fetal bovine serum were purchased from the Sigma Chemical Company (St. Louis, Mo.). [2′,3′-^{3}H]-didanosine was purchased from Moravek Biochemicals Inc. (LaBrea, Calif.). Reagent-grade chemicals, i.e., concentrated hydrochloric acid, sodium azide, and monobasic sodium phosphate (monohydrate), were purchased from Fisher Scientific (Rochester, N.Y.). Ecoscint scintillation fluid was obtained from National Diagnostics (Manville, N.J.). Knox brand gelatin (Knox Gelatin, Inc., Englewood Cliffs, N.J.) was used for the assay buffer. Dilution buffer was obtained from zidovudine radioimmunoassay kits (INCStar, Stillwater, Mich.). Assay buffer contained 0.01% gelatin and 0.02% sodium azide in 0.02 M sodium phosphate (pH 7.4).

The following working dilutions of antibodies and tracer were prepared: antididanosine rabbit antiserum, 0.15 to 0.30 mg/ml in assay buffer; goat anti-rabbit antiserum reconstituted in distilled water; and ^{3}H-didanosine, 0.0104 nCi/ml in assay buffer. Standards were made by preparing various dilutions of a 0.1-mg/ml stock solution of didanosine in assay buffer to yield concentrations of 0, 0.4, 0.8, 1.6, 2.0, 4, 6, 10, 15, 25, 50, and 100 ng/ml. Standards were prepared in dilution buffer. The standards, in 1- or 4-ml volumes, were stored in poly cryotubes at −20°C. Quality control samples were prepared from a separate 0.1-mg/ml stock solution of didanosine; dilutions were prepared in dilution buffer to attain concentrations of 2.5, 5, and 30 ng/ml, respectively. Controls were divided into aliquots and were stored in the same manner as the standards.

Radioimmunoassay. All reagents were equilibrated to room temperature before use. One hundred microliters of each standard, control, and unknown was pipetted into a borosilicate glass tube (12 by 75 mm) in duplicate. Two hundred microliters of dilution buffer was added to nonspecific binding tubes. One hundred microliters of the working solution of tracer was added to every tube. One hundred microliters of a working dilution of the primary antibody, rabbit antididanosine antiserum, was added to all tubes except the tubes used to measure total counts and nonspecific binding. All tubes were vortexed briefly, and the contents were allowed to incubate at room temperature for 2 h. One milliliter of the secondary antibody was pipetted into all tubes except those used to measure total counts, and the tubes were vortexed briefly. Excluding only the tubes used to measure total counts, all tubes were placed in a precooled centrifuge carrier and were placed in the centrifuge chamber at 4°C to incubate for 30 min. After incubation the tubes were centrifuged at 2,200 × *g* and 4°C for 1 h. The supernatant was decanted and the pellet was resuspended in 0.1 N HCl. The contents of each tube were transferred to a scintillation vial containing 5 ml of scintillation fluid. The vials were counted for 5 min in a Wallac 1409 DSA-based liquid scintillation counter (Wallac, Gaithersburg, Md.).

Quantitation. A four-parameter equation was used to fit the standard curve by using the RiaCalc.LM, version 2.65, software package (Wallac): counts per minute = $D + (A - D)/\{(1 + \exp[-B - C \cdot \ln(x)]\}$, where D had a role similar to a blank, A had a role similar to the reference, B is the intercept, and C is the slope. From this four-parameter fit, the concentrations of the controls and unknowns were generated.

Acceptance criteria. Standards within the quantitation range (2 to 50 ng/ml) were accepted only if they were within ±20% of their nominal value and their duplicate values were within 20% of each other. Standards outside the quantitation range were omitted at the discretion of the analyst, taking into account the day-to-day characteristic curve fitting. To consider a curve acceptable, at least five standards within the quantitation range must be available. Quality controls must also be within ±20% of their nominal values and must meet the duplicate criterion described above.

Method validation. (i) Standard curve fits. The calculated values for the observed standard points were evaluated for their variations and goodnesses of fit over 16 assays. For each standard, its mean value, standard deviation (SD), coefficient of variation [(standard deviation/mean) · 100], and percent true error were calculated.

(ii) Intraassay performance. Over 4 assay days a low concentration (1.00 ng/ml) and a high concentration (30.0 ng/ml) were assayed in triplicate to determine the average and range of intraassay variation and true error at these concentrations. Statistics were calculated as defined above for the standard curves.

(iii) Interassay performance. Over 16 assays the variation and true error of all controls were examined. Statistics were calculated as defined above for the standard curves.

(iv) Specificity. The following compounds (at the indicated concentrations) were tested to determine if they would cause falsely elevated didanosine concentrations: zidovudine (0.972 and 10 μg/ml), zalcitabine (0.1, 10, and 100 μg/ml), and stavudine (0.1, 1, and 100 μg/ml). The manufacturer previously tested the primary antibody for cross-reactivity with numerous endogenous substances. Plasma specimens from 22 patients who were about to begin didanosine treatment were tested to determine whether there were false-positive didanosine results. Lastly, 20 heparinized plasma samples from healthy volunteers were tested to determine whether there was nonspecific binding and compared with the nonspecific binding of the standard.

Matrix effect and assay dilution. To compare the plasma matrix with other protein matrices, assay buffer, fetal bovine serum, and plasma were spiked to

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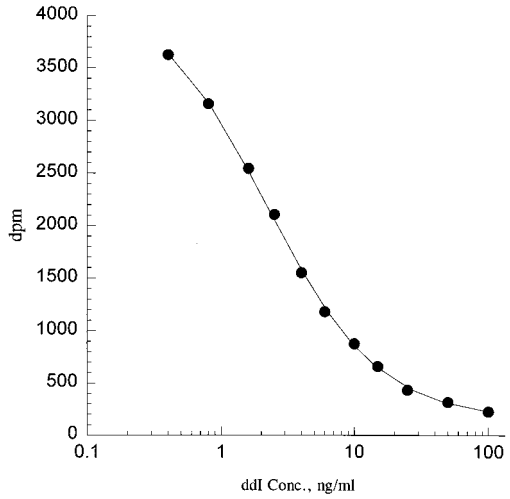

FIG. 1. Standard curve representative of a typical radioimmunoassay for didanosine. The fitted line depicts the four-parameter fit used for the assay.

contain 0.0358, 0.568, 2.88, 6.05, and 10.8 ng of didanosine per ml. The samples were assayed undiluted, and the results were compared. To further characterize plasma matrix effects, heparinized plasma from healthy volunteers was assayed after adding didanosine so that the plasma samples contained from 0.342 to 1,830 ng of didanosine per ml. Dilutions of 1:2, 1:3, 1:5, 1:10, and 1:100 were assayed, as were undiluted samples. Each dilution of sample was assayed six times. The plasma blank was also assayed to determine any background interference.

Didanosine was also measured after addition to urine, urine buffered with 0.2 M potassium phosphate buffer (pH 8.0; 1:3 and 1:10), and cerebrospinal fluid. The didanosine concentrations in all samples were measured in quadruplicate.

Several samples from a uremic patient were analyzed at different dilutions to determine if this patient population might present additional difficulties in quantitation because of the abnormal constituents often found in the plasma of uremic patients.

Stabilities of quality controls and plasma specimens. Samples were prepared in assay buffer at 1.00, 2.50, and 30.0 ng/ml as outlined above in the description of the reagents. The preparation was split into three aliquots. One aliquot was briefly kept at 4°C until it was assayed; the other aliquots were frozen at −20°C. The frozen aliquots were treated differently: one was thawed only once before assay and the other was frozen and thawed three times prior to assay. All aliquots were assayed six times, and the results were tested for significance by using one-way analysis of variance. In addition, 25 plasma specimens were reassayed for their didanosine concentrations 2.5 years after their initial analyses; these specimens had been stored at −20°C. The results were compared by a paired *t* test.

Statistical calculations. Statistical calculations were performed by using Lotus 1-2-3, version 4.0 (Lotus Development Corporation Inc., Cambridge, Mass.), spreadsheet and formula functions. Analysis of variance and *t* tests were performed by using INSTAT software, version 1.01 (GraphPAD Software, San Diego, Calif.).

TABLE 1. Statistics for the didanosine standard curve

Standard concn (ng/ml)	Mean fitted value (ng/ml)	SD (ng/ml)	Coefficient of variation (%)	True error (%)	No. of samples
0.400	0.400	0.0339	8.49	0	11
0.800	0.762	0.0618	8.11	−4.7	16
1.60	1.58	0.143	9.06	−1.1	13
2.50	2.08	0.175	8.40	+3.9	16
4.00	4.06	0.220	5.41	+1.6	14
6.00	6.18	0.375	6.07	+3.0	15
10.00	10.3	0.689	6.67	+3.2	16
15.00	13.9	0.563	4.06	−7.5	16
25.00	27.8	1.49	5.37	+11	9
50.00	50.9	3.75	7.37	−1.8	15
100.00	153	33.2	21.7	+52	16

TABLE 2. Intra-assay variability for radioimmunoassay for didanosine

Study no.	1 ng/ml				30 ng/ml			
	Values	Mean	SD	Coefficient of variation (%)	Values	Mean	SD	Coefficient of variation (%)
1	1.07	1.11	0.05	4.50	31.63	29.62	2.80	9.45
1	1.09				30.81			
1	1.16				26.42			
2	0.98	1.05	0.07	6.67	28.76	28.95	0.23	0.79
2	1.04				29.20			
2	1.12				28.89			
3	0.95	0.99	0.04	4.04	29.26	29.12	0.45	1.55
3	1.00				29.49			
3	1.02				28.62			
4	0.90	0.96	0.05	5.21	29.60	29.67	0.08	0.27
4	1.00				29.66			
4	0.98				29.76			
5	0.90	0.93	0.03	3.23	29.76	28.85	0.83	2.88
5	0.95				28.12			
5	0.94				28.67			

RESULTS

Figure 1 illustrates a representative standard curve for didanosine with a four-parameter fit. Total counts were typically 3,400 to 5,000 dpm, and nonspecific binding ranged from 10 to 40 cpm. Table 1 summarizes the variation and error averaged over 11 assays. Variation was typically 4 to 22%, with greater variation seen at the highest standard concentration. True error ranged from −5 to 52%. The fit of the standard curve was not biased because it exhibited a good scatter of positive and negative errors with respect to the range of concentrations. Table 2 displays the intra-assay results over four assays. Variation ranged from 3 to 7% at 1 ng/ml and from 0.3 to 3% at 30 ng/ml. True error ranged from −7 to 5% at 1 ng/ml and from −4 to −1% at 30 ng/ml. Table 3 provides the results for the quality controls assayed over 16 assays. Variations were 6, 7, and 11% at 2.5, 5, and 30 ng/ml, respectively. True error averages ranged from −8 to −3%; however, daily individual values did measure both above and below the target values for the quality controls. Of the compounds tested, there was no indication of interference. None of the 22 samples obtained from patients before administration of the dose tested positive for didanosine. However, the disintegrations per minute in the plasma samples were significantly different from those of the zero standard, indicating a consistent value much less than zero. Further discussion of this phenomenon follows in the plasma matrix results section. Nonspecific binding in plasma from volunteers was fairly consistent (156 ± 34.4 dpm) and compared fairly well with the nonspecific binding of the standard, averaging 130 dpm.

Tables 4 and 5 display the results of the matrix effect and

TABLE 3. Interassay variation for radioimmunoassay for didanosine[a]

Concn (ng/ml)	Mean (ng/ml)	SD (ng/ml)	Coefficient of variation (%)	True error (%)
2.5	2.42	0.137	5.66	−3.10
5	4.61	0.318	6.89	−7.75
30	28.1	2.97	10.6	−6.29

[a] A total of 16 samples were tested for each concentration.

TABLE 4. Recovery of didanosine in Plasma and recovery of other protein matrices by radioimmunoassay

Didanosine concn (ng/ml)	% Recovery							
	Assay buffer	Fetal bovine serum	Neat plasma	Diluted plasma				
				1:2	1:3	1:5	1:10	1:100
0.0358	568	172	0	—	—	—	—	—
0.342	—	—	30.3	—	—	—	—	—
0.568	79.8	112	30.5	—	—	—	—	—
1.25	—	—	63.1	—	—	—	—	—
1.52	—	—	53.7	36.0	28.8	—	—	—
2.88	87.8	90.6	63.8	—	—	—	—	—
3.07	—	—	73.9	59.0	52.8	—	—	—
4.05	—	—	75.2	67.4	68.6	54.4	20.9	—
6.05	87.8	87.7	83.5	—	—	—	—	—
6.35	—	—	70.7	73.5	62.8	—	—	—
10.0	—	—	86.2	81.0	74.1	123	114	—
10.8	91.6	110	75.88	—	—	—	—	—
27.2	—	—	76.75	—	—	—	—	—
40.0	—	—	—	—	72.8	86.5	80.3	—
50.0	—	—	72.8	83.4	88.6	90.0	92.0	—
193	—	—	—	—	—	—	—	104
1,830	—	—	—	—	—	—	—	93.5

[a] —, sample not tested.

dilution on measurement of didanosine concentrations. Table 4 reveals that didanosine concentrations were lower in plasma than other protein matrices; Fig. 2 depicts those data graphically for plasma by using the dilute didanosine concentration, showing that the recovery of didanosine is dependent on concentration rather than on the dilution used. The bias of blank plasma to give values much lower than zero may manifest itself, in part, in this manner. At assayed concentrations of 2.00 ng/ml and greater, independent of dilution factor, the concentrations in the plasma samples assayed were ≥70% of the target value. Table 5 displays the results for buffered urine. Accuracy was excellent for all dilutions except the highest dilution (1:1,000) when urine buffered 1:3 was used and was adequate when urine buffered 1:10 was used. Undiluted, blank buffered urine gave false-positive results. Unbuffered urine gave didanosine concentrations elevated 20 to 400% (data not shown); the inaccuracy was greatest at lower concentrations. Dilution of the urine sample did not improve the accuracy.

TABLE 5. Urine matrix effects on radioimmunoassay for didanosine

Sample and didanosine concn (ng/ml)	Dilution	Mean concn (μg/ml)	SD	Coefficient of variation (%)	True error (%)
Urine buffered 1:3					
0	None	1.67[a]	NA[b]	NA	NA
0	1:3	0.701[a]	NA	NA	NA
0	1:10	0.322[a]	NA	NA	NA
0	1:100	0.124[a]	NA	NA	NA
30	None	31.3	4.33	13.9	4.25
30	1:3	31.1	1.89	6.1	3.55
1,000	1:100	988	9.20	0.93	−1.20
1,000	1:1,000	970	79.5	8.23	−3.33
Urine buffered 1:10					
30	None	27.8	1.90	6.85	−7.42
30	1:3	30.3	0.441	1.46	1.10
1,000	1:100	930	95.0	10.2	−6.98
1,000	1:1,000	756	163	21.6	−24.4

[a] The mean is the average for two subjects.
[b] NA, not applicable.

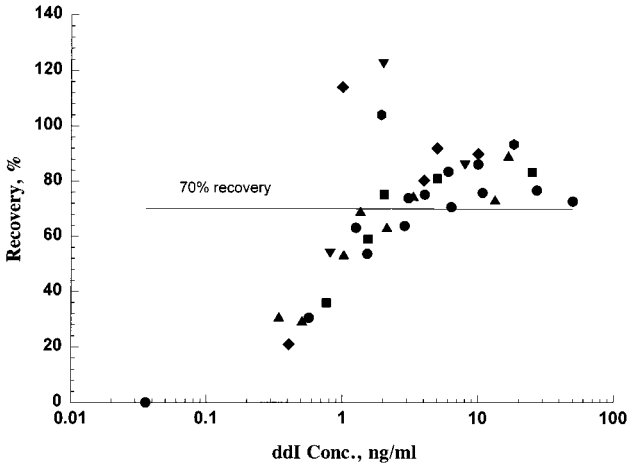

FIG. 2. Data from percent recovery experiments showing that recovery of didanosine is dependent on the in vitro concentration. Specimens containing 2 ng/ml or greater exhibit accuracy within ≥30% (recovery, ≥70%). Recovery is not dependent on dilution. ●, neat; ■, 1:2; ▲, 1:3; ▼, 1:5; ◆, 1:10; ●, 1:100.

Undiluted cerebrospinal fluid yielded good recovery at 12.5 ng/ml: 13.3 ± 0.719 ng/ml. The result for the blank matrix was below the sensitivity of the assay. The results for plasma from uremic patients are provided in Table 6. Quantitation of didanosine in plasma from uremic patients was significantly lower when plasma specimens were assayed undiluted.

No statistical significance was found between the three groups of samples (not frozen, frozen and thawed once, and frozen and thawed thrice) at any of the control concentrations. No trends in measured differences because of storage or age were found for plasma samples stored at −20°C for 2.5 years. The paired *t* test was not significant ($P = 0.82$). One outlier was excluded from statistical testing because the pair's relative difference was greater than 3 standard deviations outside the mean relative differences for the remaining 25 observations.

DISCUSSION

With the increased use of didanosine, it is apparent that many patients develop clinical intolerance because of either gastrointestinal discomfort, peripheral neuropathy, or pancreatitis, which requires dose reduction. Another reason for the use of lower dosages of didanosine has been the use of combination regimens with other antiretroviral agents such as zidovudine (3, 5, 14, 15, 18). Thus, the clinical use of lower daily doses combined with the short half-life of didanosine in plasma necessitates that assay methods with sensitivities greater than those of the available HPLC assays be developed

TABLE 6. Effect of dilution on didanosine concentrations in plasma of uremic patients

Specimen no.	Didanosine concn (ng/ml) for the following dilution factor:		
	Neat	3	10
1	37.7	99.0	85.2
2	24.6	83.2	101
3	24.0	34.9	37.1
4	41.6	93.6	103

for the conduct of traditional and population pharmacokinetic studies (11).

The didanosine radioimmunoassay is able to measure concentrations as low as 2.0 ng/ml in plasma, urine, and cerebrospinal fluid. We have used this assay for traditional pharmacokinetic studies as well as population pharmacokinetic studies (1, 13, 16). An important area for further clinical research by this assay is the use of didanosine in patients with reduced renal function. In fact, the package insert for didanosine does not provide dosing recommendations for patients with reduced renal function (4). The radioimmunoassay can be used to measure didanosine levels in the plasma of uremic patients; however, a dilution of 1:3 is needed. Interestingly, it appears that the plasma of uremic patients may contain a didanosine-binding protein which leads to a false low concentration if the sample is measured undiluted. This is in contrast to plasma from individuals with good renal function, whose samples may be assayed undiluted.

In summary, the didanosine radioimmunoassay may have direct clinical application for human immunodeficiency virus-infected patients receiving didanosine. Didanosine has a wide range of bioavailabilities, leading to considerable interpatient differences in drug exposure from the currently prescribed fixed-dosage regimens (2, 7). Some investigators have noted a relationship between concentrations in plasma and neurologic development in children and the development of pancreatitis or peripheral neuropathy (2, 8). The radioimmunoassay may be useful in examining these issues further, with the hope of attaining a more individualized approach to chronic didanosine treatment.

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