Determination of stavudine in human plasma and urine by high-performance liquid chromatography using a reduced sample volume

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Abstract

Sensitive high-performance liquid chromatographic assays have been developed for the quantification of stavudine (2′,3′-didehydro-3′-deoxythymidine, d4T) in human plasma and urine. The methods are linear over the concentration ranges 0.025–25 and 2–150 µg/ml in plasma and urine, respectively. An aliquot of 200 µl of plasma was extracted with solid-phase extraction using Oasis® cartridges, while urine samples were simply diluted 1/100 with HPLC water. The analytical column, mobile phase, instrumentation and chromatographic conditions are the same for both methods. The methods have been validated separately, and stability tests under various conditions have been performed. The detection limit is 12 ng/ml in plasma for a sample size of 200 µl. The bioanalytical assay has been used in a pharmacokinetic study of pregnant women and their newborns. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Stavudine (2′, 3′-didehydro-3′ deoxythymidine, d4T, Zerit®) is a thymidine analogue with in vitro and in vivo activity against the human immunodeficiency virus (HIV). It is a reverse transcriptase inhibitor whose mode of action is similar to other nucleoside analogues. It is active at concentrations that are generally 100-fold below those which are cytotoxic. Following phosphorylation by cellular kinases, d4T- triphosphate is produced, which preferentially inhibits HIV-1 reverse transcriptase activity.

Maternal–infant transmission is the primary means by which young children become infected with the HIV-1 virus. Many factors have been investigated for their potential role in the prevention of mother-to-child transmission of HIV. There is an increasing evidence that the antiretroviral treatment of the infected women during gestation and delivery is an important factor for reducing the risk of HIV transmission to the infant [1–4].

Stavudine was not teratogenic in the studies performed [5]. The transfer of stavudine across the placenta was investigated using an ex vivo term placenta model and results indicate that stavudine crosses from maternal to fetal circulation by simple

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diffusion [6,7]. So, this drug could be useful by reducing the risk of maternal–infant HIV transmission.

We wanted to study the pharmacokinetics of stavudine in pregnant women and their newborns, and a method for the quantification of stavudine with a reduced sample volume and with enough sensitivity to perform the pharmacokinetic study was needed. Several methodologies have been previously reported in the literature [8–10], but the sample volume of these techniques was not suitable for our study.

In this paper we describe the development and validation of a simple and sensitive HPLC method for the quantification of stavudine in urine and following solid-phase extraction of 0.2 ml of plasma.

2. Experimental

2.1. Chemicals

Stavudine (2′,3′-didehydro-3′-deoxythymidine, d4T) and the internal standard (tymidine oxetane) were obtained from Bristol-Myers Squibb (Syracuse, NY, USA). Deionized water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Solid-phase extraction cartridges (Oasis®, 1 ml, 30 mg) were obtained from Waters (Franklin, MA, USA). HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). All other reagents were purchased from Merck. Blank plasma was obtained form the Laboratory of Blood Transfusion Service of the Hospital Clinic (Barcelona, Spain).

2.2. Instrumentation

The HPLC system consisted of the following components: a Hewlett-Packard (Palo Alto, CA, USA) Model HP1100 quaternary pump, a HP1100 degasser, a HP1100 autosampler, a HP1100 UV detector operating at 266 nm and a HP CHEMSTATION software integrator.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–10 mM potassium phosphate with 1% of

triethylamine added, adjusted to pH 2.5 with ortofosforic acid (3:97, v/v). The elution conditions were isocratic, and the mobile phase flow-rate was set at 1 ml/min. The analytical column was a Nova Pak C_{18} 5 μm particle size, 150×3.9 mm (Waters) with a guard column Nova pak C_{18} (Waters).

The sample injection volume was 40 μl for the plasma samples, and 100 μl for the urine samples. UV absorbency at a wavelength of 266 nm was used for detection. The retention times of stavudine and internal standard were 8.5 and 12 min, respectively.

2.4. Preparation of standards

A stock d4T solution of 1.5 mg/ml was prepared in methanol. Serial dilutions of the stock solution with Milli-Q water, led to solutions of 10 to 750 μg/ml. Calibration standards in plasma covering the concentration range between 25 and 2500 ng/ml were prepared by adding appropriate volumes of these solutions to drug-free plasma. Seven calibration concentrations were used to define the standard curve (25, 50, 100, 250, 500, 1500, 2500 ng/ml). Calibration standards in urine were prepared similarly, adding appropriate volumes of d4T solutions to drug-free urine covering the concentration range between 2 and 150 μg/ml (2, 10, 25, 50, 75, 100 and 150 μg/ml).

A second stock solution of d4T of 1.5 mg/ml was used for the preparation of quality control standards in plasma (30, 150 and 1000 ng/ml) and in urine (10, 50 and 100 μg/ml). All calibration and quality control standards were divided into polypropylene microtubes as 250-μl aliquots, and frozen at −80°C until assay.

A stock solution of internal standard of 1 mg/ml was prepared in methanol and diluted to 100 μg/ml in 50% methanol for use during sample preparation.

2.5. Sample pretreatment

Blood samples were collected in tubes with potassium-EDTA, and centrifuged (10 min., 2000 g) as soon as possible. Serum was decanted and heated for 30 min at 56°C to inactivate HIV virus, before storing at −80°C. Urine samples were not deactivated.

2.6. Sample preparation

2.6.1. Plasma assay

Solid-phase extraction cartridges (Oasis®, 1 ml, 30 mg) were conditioned sequentially with methanol (1 ml) and water (1 ml). An aliquot of 200 μl of the plasma sample was added to the cartridge and was allowed to pass through the bed with minimal suction. Two 1-ml water aliquots were added to the cartridge. The cartridge bed was suctioned dry. Stavudine was eluted with 1 ml of methanol. The eluent was evaporated to dryness under a nitrogen stream at ambient temperature, the residue was reconstituted with 50 μl of mobile phase, and then 40 μl were injected onto the HPLC system.

2.6.2. Urine assay

An aliquot of 10 μl of the urine sample, was placed on a microtube, 20 μl of the internal standard (tymidine oxetane, 100 μg/ml) were added, followed by 970 μl of HPLC water. 100 μl of this dilution were injected onto the HPLC system.

2.7. Calibration and calculation procedures

2.7.1. Plasma assay

Daily standard curves were constructed using the peak height of stavudine. The unknown concentrations were computed from the unweighted linear regression equation of the peak height against concentration of stavudine in ng/ml.

2.7.2. Urine assay

Daily standard curves were constructed using the ratio of the observed peak height of stavudine and the internal standard. The unknown concentrations were computed from the unweighted linear regression equation of the peak height ratio against concentration of stavudine in μg/ml.

2.8. Accuracy, precision and recovery

The accuracy and intra-day and inter-day precision of the method were estimated by assaying five replicate plasma or urine samples at three different concentrations, in three analytical runs.

The overall mean precision was defined by the coefficient of variation (C.V.) with relative errors from five standards of three different concentrations analyzed on the same day. Inter-day variability was estimated from the analysis of the five standards on three separate days. Recovery of stavudine after the solid-phase extraction was determined by comparing observed stavudine concentration in extracted plasma, to those of non-processed standard solutions.

2.9. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank matrices. Potential antiretroviral agents used in combination therapy with stavudine including other reverse transcriptase inhibitors (AZT, 3TC, ddI, ddC, nevirapine) and protease inhibitors (indinavir, ritonavir, saquinavir and nelfinavir) were also analyzed and were verified to be chromatography resolved from stavudine and internal standard.

2.10. Stability

HIV-infected patient samples are routinely heated at 56°C to inactivate the virus prior to handling. Heat deactivation studies were performed to verify the stability of d4T in plasma under this conditions. An additional stability test was performed to verify the stability of d4T (and internal standard) in the injectate while waiting for HPLC analysis. The samples were left at room temperature for 24 h. The stability of d4T during sample handling was also verified subjecting samples to three freeze–thaw cycles.

3. Results

3.1. Linearity

3.1.1. Plasma

Peak height of d4T values of calibration standards were proportional to the concentration of d4T in plasma over the range tested (25–2500 ng/ml). The calibration curves were fitted by linear least-squares regression and showed coefficients of determination greater than 0.999. The mean slope was 0.01749 with an SD of 0.00102, and a C.V. of 5.8%. The mean y-intercept was 0.02633.

3.1.2. Urine

Peak height ratio values of calibration standards were proportional to the concentration of d4T in urine over the range tested. The calibration curves were also fitted by linear least-squares regression and showed coefficients of determination greater than 0.999. The mean slope for the urine curve (2–100 µg/ml) was 0.006030 with an SD of 0.0003719 and a C.V. of 1.6%. The mean y-intercept was of 0.007628.

3.2. Selectivity

We analyzed six different blanks of plasma and urine from each of six different individuals. Representative chromatograms of blank and spiked plasma and urine samples are illustrated in Figs. 1 and 2, respectively. Blank plasma showed no interfering endogenous substances with d4T but a endogenous substance which appeared in the six blanks tested, did interfere with the I.S., this is the reason for not

using an I.S. in plasma. The linearity of the method without the I.S. was also very good. Blank urine showed no interfering compounds neither with d4T nor with the I.S.

Potentially coadministered drugs tested had retention times that were different from d4T or the I.S. or were not detected with the described bioanalytical method.

3.3. Limit of detection and limit of quantification

The estimation of the detection limit and the quantification limit was based on the standard deviation of the response and the slope of the calibration curves.

The limit of detection (LOD) and the limit of quantification (LOQ) could be defined as

$$LOD = \frac{X + 3\sigma}{X} \quad LOQ = \frac{X + 10\sigma}{S}$$

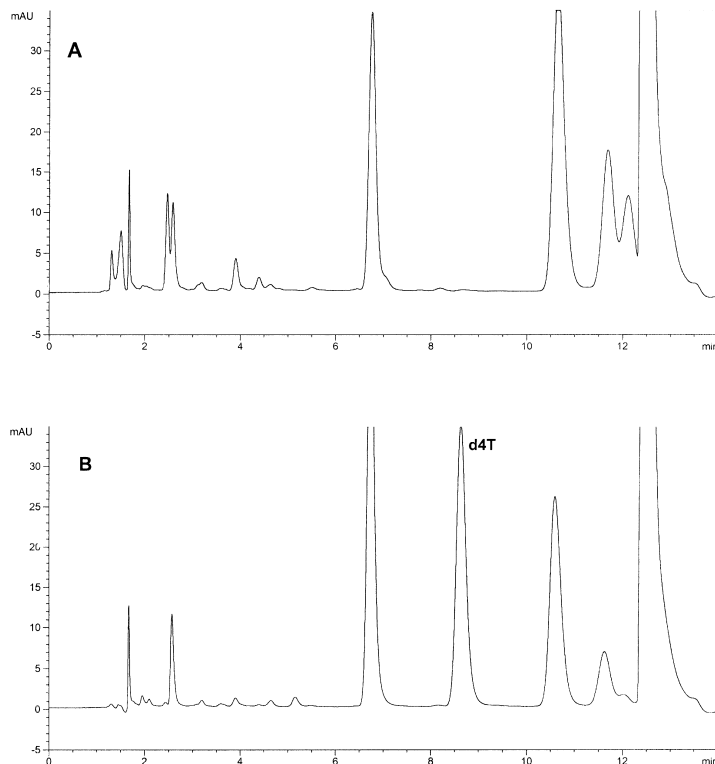where $X =$ the mean response of the matrix blanks

Fig. 1. Chromatograms of blank plasma (A) and blank plasma spiked with 500 ng/ml of stavudine (d4T) (B).
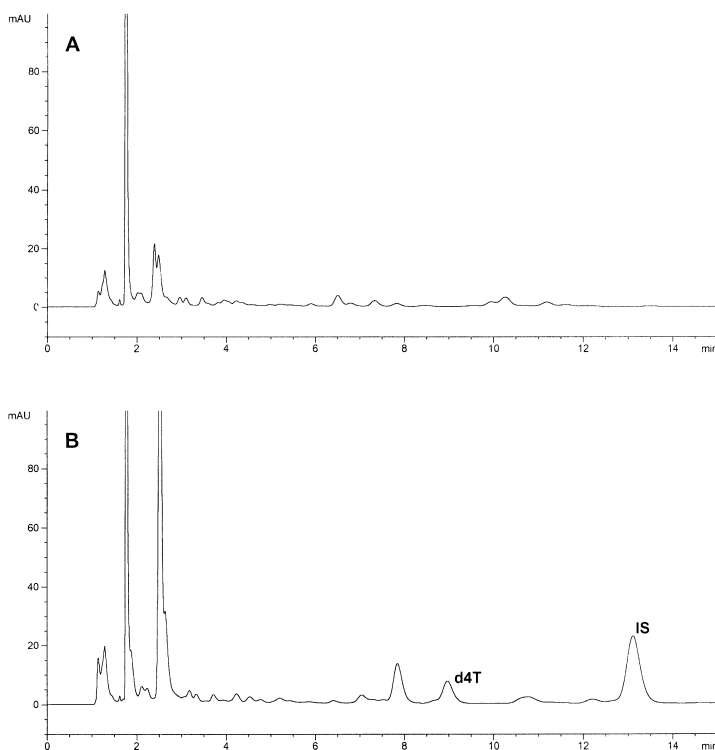
Fig. 2. Chromatograms of blank urine (A) and blank urine spiked with 20 μg/ml of stavudine (d4T) and 100 μg/ml of the internal standard (I.S.) tymidine oxetane (B).

($n=6$), $\sigma=$ the standard deviation of the response and $S=$ the slope of the calibration curve.

We analyzed six different blanks of plasma and six blanks of urine, and we found the mean response of the blank and the standard deviation of the response in each matrix. The results of the limits of detection and of quantification for plasma and urine are shown in Table 1.

3.4. Accuracy, precision and recovery

The results from the validation of the method in human plasma and urine are listed in Tables 2 and 3, respectively. This results indicate that both methods are accurate (average accuracy ranged from 94 to 110%), and precise (within-day precision ranged from 1 to 4.9% and between-day precision from 3.3 to 10%). The mean recovery of the method is $80\pm5.74\%$.

3.5. Stability

The stability of stavudine under various conditions is shown in Table 4. Under all conditions tested stavudine is stable with concentrations of at least 93.6% of the initial concentration.

Table 1
Limits of detection and quantification in plasma and urine

Matrix	Mean baseline response	SD response	LOD	LOQ
Plasma	0.1019	0.03122	11.6 ng/ml	24.6 ng/ml
Urine	0.0064	0.00055	1.33 μg/ml	1.97 μg/ml

Table 2
Accuracy and precision of the determination of stavudine in plasma

	Theory (ng/ml)	n	Found±S.D (ng/ml)	C.V. (%)	Accuracy (%)	Recovery (%)
Intra-day	30	5	28.26±1.38	4.89	94.21	81.0
	150	5	153.16±6.011	3.92	102.10	73.5
	1000	5	990.01±43.76	4.42	99.01	85.4
Inter-day	30	15	32.22±3.38	10.47	107.41	–
	150	15	150.84±8.272	5.48	100.56	–
	1000	15	1017.33±51.06	5.02	101.73	–

Table 3
Accuracy and precision of the determination of stavudine in urine

	Theory (µg/ml)	n	Found±S.D (µg/ml)	C.V. (%)	Accuracy (%)
Intra-day	10	5	11.19±0.112	1.00	111.18
	50	5	48.01±0.771	1.60	96.02
	100	5	99.80±1.670	1.67	99.80
Inter-day	10	15	10.67±0.431	4.04	106.73
	50	15	50.69±2.501	4.93	101.37
	100	15	97.97±3.242	3.30	97.97

4. Conclusion

The plasma and urine assay methods described herein are currently being used for the assay of clinical study samples. Examples of plasma concentration versus time and cumulative excretion profiles in a pregnant woman treated with an oral dose of 40 mg of stavudine are depicted in Fig. 3. The maximum concentration of stavudine, reached 90 min after the drug intake, was 876.3 ng/ml.

Within 6 h, the urinary excretion was almost complete, over a 12-h period the 35% of the oral dose was excreted as the parent compound.

In conclusion, a simple, validated assay, that can readily be used in any laboratory, for the quantitative determination of stavudine in human plasma and urine is described. The assay covers the concentration range of interest and, due to the reduced sample volume used is suitable for pharmacokinetic studies in HIV infected pediatric population.

Table 4
Stability of stavudine in spiked human plasma samples

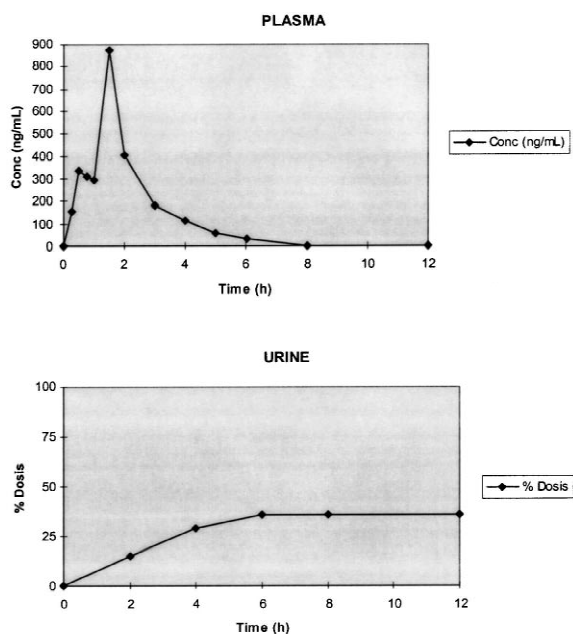| Storage conditions	Concentration (ng/ml)	Recovery (%)	C.V. (%)	n
30 min at 60°C	30	102.1	6.5	5
	150	101.1	7.2	5
	1000	94.8	5.9	5
24 h at 25°C	30	93.6	3.7	5
	150	94.5	4.2	5
	1000	98.16	4.5	5
Three freeze–thaw cycles	30	98.5	4.8	5
	150	103.8	3.3	5
	1000	100.3	2.2	5

PLASMA

URINE

Fig. 3. Plasma concentration versus time and cumulative excretion profiles in an adult pregnant woman treated with a 40-mg oral dose of stavudine.

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