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Development and validation of a reversed-phase liquid chromatographic method for analysis of D4T (Stavudine) in rat plasma

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Abstract

An improved and simplified high-performance liquid chromatographic (HPLC) method at UV detection 265 nm is presented for the determination of d4T in rat plasma. The mobile phase consists of methanol–distilled water–acetic acid in the 23:77:0.2 (v/v) ratio. Quantification is achieved by the peak-area ratio method with reference to the internal standard. This paper presents linearity, accuracy, precision, limit of quantification and limit of detection, specificity–selectivity and sample stability data. Based on the intra and inter-day validation, all coefficients of variation (CV) were found less than 15%. The assay is sufficiently rapid and sensitive and was applied in a pharmacokinetic study in rats.

Keyword: Stavudine

1. Introduction

2′,3′-Didehydro-3′-deoxythymidine (D4T) is a powerful dideoxynucleoside analogue which has shown powerful activity against human immunodeficiency virus (HIV) [1]. It Inhibits replication of HIV inhibiting reverse transcriptase activity with relatively little inhibition of host cell DNA polymerases in various cell types [3].

Analytical methods have been reported for the analysis of d4T in biological fluids. Specific competitive ELISA method was applied to the measurement of d4T in plasma from HIV seropositive-treated patients and to quantification of the intracellular and unmetabolized drug in CEM and Molt 4 cell lines [2,3]. The assay utilized specific antibodies for d4T. HPLC methods with complex and large sample preparation procedure has been used in pharmacokinetic study [4–11].

In this work, we have developed and validated a sensitive and precise HPLC method for the quantitative determination of d4T in rat plasma, incorporating an internal standard, using an isocratic reverse-phase system for the separation, a small sample volume for a short and simple sample preparation procedure. This method was also applied for pharmacokinetic study in rats Sprague Dawley (SD).

2. Experimental conditions

2.1. Chemicals and reagents

Methanol (HPLC grade) and perchloric acid were obtained from BDH (Poole, UK). d4T and 3′,5′-anhydrothymidine (internal standard) were obtained from Organic Chemistry Synthesis Laboratory of the National Center for Scientific Research. Demineralized and double-distilled water was prepared in our laboratory. Rat plasmas were drawn in our laboratory from SD rats.

2.2. Apparatus and HPLC-conditions

The chromatographic system consisted of 2150 HPLC pump (Pharmacia), PU4020 variable wavelength UV detector (PYE UNICAM) to detect absorbance at 265 nm and 7125 injector (Rheodyne) with a 20 μl loop. A stainless

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steel Hibar column (125 mm × 4 mm i.d.) prepackaged with 5 μm LiChrospher 100 RP-18 (Merck) was used. The mobile phase consisted of methanol–distilled water–acetic acidic 23:77:0.2 (v/v). The flow rate was 1 ml/min. Peak-areas were acquired by EZChrom Chromatography Data System. All analyses were performed at room temperature.

2.3. Sample preparation

Stock solutions of d4T and 3′,5′-anhydrothymidine (internal standard) were prepared in water (1 mg/ml). Working d4T standard solutions were prepared at concentrations of 5, 20, 30, 60, 70 and 90 μM and a working internal standard solution was prepared at 45 μM. The standard solutions were stored at 4 °C. Quality control samples (QCs) were prepared at concentrations of 10, 55 and 80 μM.

d4T-Free plasma was obtained from rat Sprague Dawley (SD). Plasma samples were kept deep frozen (−20 °C) until analysis and were thawed at room temperature. 300 μl blank plasma was taken into a microcentrifuge Eppendorf tube and added 15 μl working standard solution of d4T and 15 μl internal standard solution. Plasma proteins were precipitated by addition of 330 μl of 10% perchloric acid solution. The mixture was shaken for 30 s (Vortex, Heidolph REAX 2000, Germany). The samples were centrifuged at 3000 × *g* for 10 min in a microfuge (SIGMA 112, Germany), the supernatant was transferred to microcentrifuge Eppendorf tube and 50 μl was injected directly into the chromatographic system.

2.4. Validation of analytical method

2.4.1. Linearity and quantification limit

Standard curve data were generated by spiking a series of drug-free plasma samples with d4T to produce concentration ranges of 0.8–90 μM. Extraction procedure and analysis were carried out as described above. Calibration curve was constructed by plotting the ratios of peak areas against concentration and analyzed by linear regression analysis.

The quantification limit (LOQ) is the lowest amount of an analyte which can be quantitatively determined with defined precision and accuracy under the given experimental conditions. The accuracy and precision of LOQ was evaluated experimentally, preparing and analyzing blank of plasma spiked with d4T at concentration of 0.80 μM.

2.4.2. Accuracy

The accuracy was determined by analysing 3 parallels of QCs (at concentration of 10, 55 and 80 μM.)

2.4.3. Precision

Intra-day variation was determined by analysing 3 parallels of QCs. (at concentration of 10, 55 and 80 μM.)

Inter-day variation was determined at each QCs (concentration) on three different days.

2.4.4. Extraction efficiency

The recovery of d4T and IS were determined. The recovery of d4T was determined for QCs (at concentration of 10, 55 and 80 μM) and for IS was determined at concentration of 45 μM. Three replicates of each QCs were extracted by the above mentioned sample preparation and injected into the HPLC system. Three replicates of each QCs prepared in aqueous solutions were directly injected. The extraction recovery at each concentration was calculated using the following equation:

$$\text{Recovery} = \frac{\text{peak area after extraction}}{\text{peak area after direct injection}} \times 100$$

2.4.5. Specificity

The specificity was determined by analysis of blank plasma and added plasma with d4T and IS.

2.5. Stability

In order to assess the stability of d4T, fresh rat samples spiked with a known amount of d4T (QCs) were stored at 4 and −20 °C and assayed until a week.

2.6. Pharmacokinetic study

The study was conducted in accordance with The Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research. Forty five fasted male Sprague Dawley (SD) (body weights, 230–250 g) were administered by intravenuos (i.v.) administration of 10 mg/kg in aqueous solution. Rats were divided into nine groups ($n = 5$) based on the time of blood sampling having five animals each. Blood samples (3 ml) were colected using dried heparinized tubes at 0, 15, 30, 45, 60 and 120 min after dosing. The blood was centrifuged at 3000 × *g* during 10 min and the plasma was frozen at −20 °C and stored until analysis.

2.7. Pharmacokinetic analyses

Pharmacokinetic modeling and parameter calculations were carried out using the pharmacokinetic software PKCALC [11]. Noncompartimental analysis and parameter calculations were carried out using logarithmic data of the plasma concentration–time profile. The predicted area under the concentration–time curve ($AUC_{0–120}$) was calculated based on the linear trapezoidal method.

3. Results

The resulting chromatograms were essentially free from endogenous interference (Fig. 1). The retention times of d4T and internal standard were 2.98 and 3.92 min, respectively.

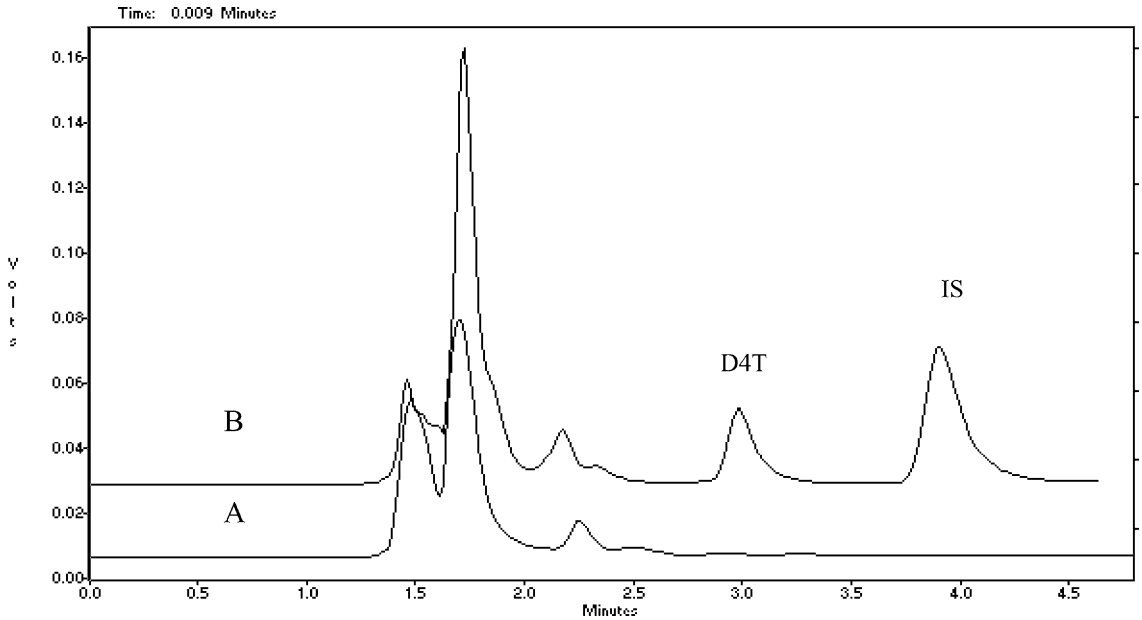

Fig. 1. Chromatograms obtained for specific of method: (A) blank plasma; (B) blank plasma spiked with 10 μM of d4T (2.98 min) and 45 μM of IS (3.92 min).

The peak shapes and the resolution between d4T and internal standard was satisfactory or suitable.

The linearity of the calibration curve was determined by plotting the drug concentration against ratio of drug/internal standard peak area. The equation of the line ($y = 0.015x + 0.0009$) and correlation coefficient (r) and determination coefficient (r^2) of calibration curve were 0.9964 and 0.9927, respectively. Concentrations range is higher than other research reports [12]. In our study, the range is optimal to determine the expected concentrations or should appear for D4T in plasma for the dose (10 mg/kg) evaluated in rats. The calibration curve showed excellent linearity over the range of concentrations examined (0.8–90 μM). The limit of quantification limit (LOQ) was 0.80 μM. The accuracy and precision of quantification limit was evaluated experimentally obtaining LOQ 0.82 ± 0.04 μM with 98–106% recovery and coefficient of variation (CV) of 4.6% (Table 1).

Accuracy was assessed by meaning of recovery; concentration found and concentration added were evaluated (Table 2). We have obtained relationship between concentration found and concentration added. The equation of the line was ($y = 1.051x - 0.068$) for correlation coefficient (r) and determination coefficient (r^2) of curve were 0.999 and 0.997, respectively.

Table 1
Experimental evaluation of LOQ

Concentration added (μM)	Concentration found (μM)	Recovery (%)
0.80	0.85	106.25
	0.85	106.25
	0.78	97.50
Mean ± S.D.	0.82 ± 0.04	103.33 ± 5.05
CV (%)	4.67	4.89

Precision of the assay procedure was assessed calculating the intra-and-inter day variation for each concentration. The intra-day variability is presented in Table 3 and inter-day variability of the assay on three different days is presented in Table 4. All intra-and-inter-day coefficients of variation (CV) were less than 15%.

The extraction recoveries of d4T and IS in plasma are presented in Table 5. All recoveries were found more than 99%.

The stability of d4T in plasma at 4 and −20 °C was satisfactory. For a week no significant reduction of the plasma concentration was observed.

Table 2
Accuracy of method

Concentration added (μM)	Concentration found (μM) (mean ± S.D.) ($n = 3$)	CV (%)	Recovery d4T (%), (mean ± S.D.) ($n = 3$)	CV recovery (%)	Recovery IS (%) (mean ± S.D.) ($n = 3$)
10	9.71 ± 0.52	5.36	97.13 ± 5.24	5.39	99.21 ± 1.12
55	55.01 ± 0.61	1.11	100.02 ± 1.30	1.30	100.10 ± 0.51
80	83.02 ± 1.19	1.43	103.78 ± 1.48	1.43	98.89 ± 2.30

Table 3
Intra-day variation of d4T in the analysis of rat plasma

Concentration added (μM)	Concentration found (μM) (mean ± S.D., $n = 3$)	CV (%)
10	9.60 ± 0.69	7.20
55	54.60 ± 2.27	4.20
80	82.20 ± 0.53	0.60

Table 4
Inter-day variation of d4T in the analysis of rat plasma

Concentration added (μM)	Concentration found (μM), (mean ± S.D., $n = 9$)	CV (%)
10	10.41 ± 0.62	5.99
55	55.67 ± 1.22	2.19
80	83.28 ± 2.32	2.79

Mean plasma concentration–time profile of d4T obtained after intravenous administration of 10 mg/kg in rat is presented in Fig. 2 and the pharmacokinetic parameters obtained are shown in Table 6.

Plasma concentration profile in rats after received d4T (10 mg/kg) as intravenous bolus decreased monophasically (Fig. 2) and were measurable from 5 to 120 min on average from 15.61 to 0.69 μg/ml. After intravenous administration the volume of distribution was extensive (Vd = 207.03 ml). This, together with the elevated values total clearance (CL = 5.30 ml/min), half life ($t_{1/2}$ = 25.94 min) and mean residence time (MRT = 40.14 min) indicates that d4T rapidly and extensively leaves central circulation. The present results are consistent with those previously observed by Toshiyuki et al. [13]. These authors found a similar plasma concentration time profile after iv administration of similar doses of d4T (44.6 μmol/kg) to rats and therefore, similarities in area under curve (AUC = 450.66 μg min/ml) and $t_{1/2}$ (36 min) were observed. The fact that a very closed plasma concentration time profile has been observed using different

Table 6
Pharmacokinetic parameters obtained after intravenous administration of 10 mg/kg d4T in rat

$t_{1/2}$ (min)	25.94
k_e (min^{-1})	0.0267
CL (ml/min)	5.30
Vd (ml)	207.03
MRT (min)	40.14
AUC_{0-120} (μg min/ml)	474.35
AUC (μg min/ml)	497.27

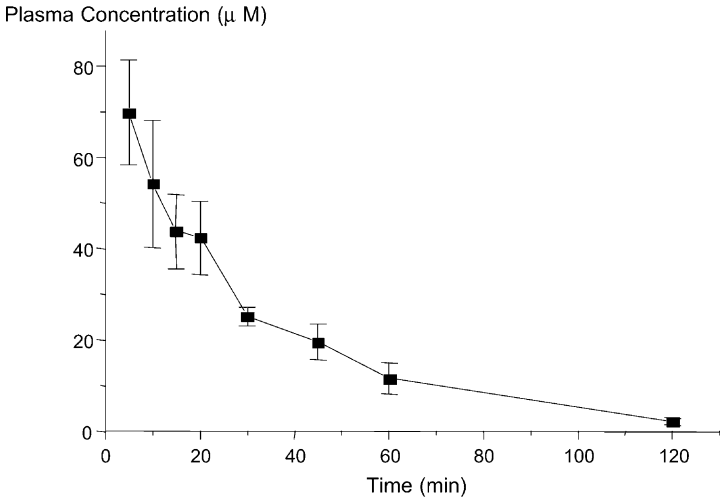

Fig. 2. Mean plasma concentration–time profile of d4T obtained after intravenous administration of 10 mg/kg in rat.

Table 5
Extraction efficiency obtained for d4T and IS

Plasma concentration of d4T (μM)	Recovery of extraction (%), (mean ± S.D., $n = 3$)	Plasma concentration of IS (μM)	Recovery of extraction (%)
10	99.54 ± 5.70		100.24
55	99.23 ± 6.42	45	100.30
80	101.46 ± 4.04		100.20
Mean ± S.D.	100.08 ± 1.21	Mean ± S.D., ($n = 3$)	100.25 ± 0.05
CV (%)	1.21	CV (%)	0.05

methodologies also support the validity of our methods and its applicability in pharmacokinetic studies in rats.

4. Conclusions

A fast and simple HPLC method for the determination of d4T in rat plasma is presented. The method's linearity, precision, accuracy and recovery were excellent. Further, the method has been shown to be specific and selective. The small sample volume and sensitivity limit of $0.82 \pm 0.04\ \mu M$ makes this assay a suitable choice for pharmacokinetic studies in rats. This analytical method has been applied to pharmacokinetic studies in rat.

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