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Determination of delavirdine in very small volumes of plasma by high-performance liquid chromatography with fluorescence detection

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Abstract

Delavirdine is a newly developed anti-HIV-1 drug for AIDS therapy. This study describes a sensitive high-performance liquid chromatographic method for the determination of delavirdine in 50 μl of plasma. Samples were deproteinized with 150 μl of a solution of internal standard (cisapride 10 μg/ml) in acetonitrile. An aliquot of the supernatant was injected onto the column. HPLC separation was achieved on a C_{18} column with the mobile phase of acetonitrile-50 m*M* sodium dihydrogen phosphate (60:40, v/v) at a flow-rate of 1 ml/min. The eluants were measured by fluorescence detection with excitation at 295 nm and emission filtration at 425 nm. The retention time was about 5.3 min for delavirdine and 6.5 min for cisapride. The specificity was demonstrated, as there were no interferences from plasma samples of different batches in the regions of peak interest. Calibration curves were linear from 25 to 25000 ng/ml. The limit of quantitation was 25 ng/ml. The within- and between-day precision (C.V.) was 9.3%, or less, and the accuracy was within 9.2% of the nominal concentration. The small sample volume needed is especially advantageous for the application both in pharmacokinetic studies in HIV-infected adults and pediatric patients, and in small animals, where limited samples are available.

Keywords: Delavirdine

1. Introduction

Delavirdine mesylate (Rescriptor®, Fig. 1) is a bisheteroarylpiperazine non-nucleoside reverse transcriptase inhibitor (NNRTI) of the human immunodeficiency virus type 1 (HIV-1). It is currently used in the treatment of Acquired Immune Deficiency Syndrome (AIDS) in combination with other anti-retroviral agents in highly active anti-retroviral therapy [1–3]. In recent years, concerns regarding protease inhibitor-related metabolic disturbances have led to significant shifts in treatment practices in HIV infection towards the NNRTI-based regimens [3].

Delavirdine is rapidly absorbed and extensively metabolized after oral administration. In healthy subjects and HIV-infected patients, the average steady-state peak concentration after oral administration of delavirdine 600 mg twice daily or 400 mg three times daily is around 8.7–17 μg/ml [1,2,4].

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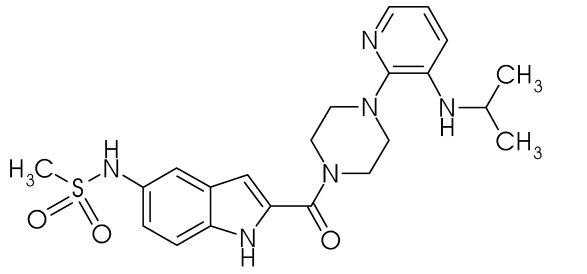
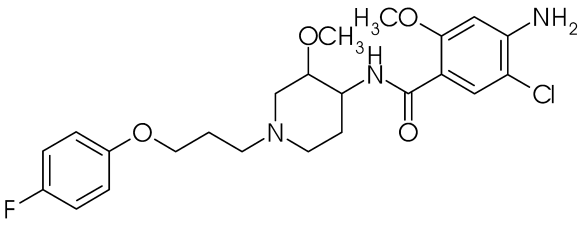


Delavirdine



Cisapride

Fig. 1. Structures of delavirdine and internal standard cisapride.

Although delavirdine is extensively metabolized to several inactive metabolites, delavirdine itself is the major component in plasma after oral dosing in rats and humans, and only the desisopropyl-delavirdine metabolite exists in significant amount in plasma [4,5]. Metabolism of delavirdine is mainly catalyzed by CYP3A with minor contribution of CYP2D6 [1,2]. Delavirdine is a potent inhibitor of CYP 3A4, and has the potential to inhibit the metabolism of itself and concomitant medicines, especially the HIV protease inhibitors. Hence pharmacokinetic interactions between delavirdine and other concomitant medicines are likely to occur [1,2]. It has been suggested that pharmacokinetic monitoring of anti-HIV therapy could provide treatment benefit by avoiding non-adherence, drug–drug interactions and resistance development [6].

Several high-performance liquid chromatography (HPLC) methods have been reported for the determination of delavirdine alone or together with its metabolites in plasma/serum using ultraviolet [6–8] or fluorescence detection [9–11]. All these methods are validated and adapted for pharmacokinetic investigations in adults using 200–1000 μl of plasma or serum. The volume is too large for studies in children and small animals, where the blood volume collected should be kept to a minimum. Hence, the pharmacokinetics of delavirdine has not been investigated in young children so far, and dosing regimens in this population have not been established and are based on empirical extrapolation from adult studies. Whereas kinetic studies in small animals, such as mice and rats, are performed using radiolabelled delavirdine that is not readily available [5].

Given the increasing worldwide epidemic of AIDS and clinical importance of delavirdine, a sensitive method for the determination of delavirdine requiring only a small sample amounts is desired. Here, we describe a simple, rapid and robust HPLC method suitable for the determination of delavirdine in plasma. Only 50 μl of plasma is required for detection and advantage is taken of the native fluorescence of delavirdine and the internal standard (cisapride, Fig. 1), which provides a high degree of sensitivity and selectivity. The method enables precise and accurate quantitation of delavirdine in very small sample volumes over a wide concentration range. The application of this method to a pharmacokinetic study in rats is presented.

2. Experimental

2.1. Chemicals and reagents

Delavirdine mesylate and cisapride were purchased from Biomol Research Laboratories (Lot P4251, Plymouth Meeting, PA, USA) and Sigma (St. Louis, MO, USA), respectively, and were used without further purification. Sodium dihydrogen orthophosphate, orthophosphoric acid (85%) and sodium hydroxide were analytical grade and were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified through a Milli-Q reagent water system (Millipore, Bedford, MA, USA). Drug-free (blank) human plasma from individual volunteers was from the Blood Bank of National Cheng Kung University Hospital (Tainan, Taiwan).

2.2. Instrumentation and chromatography

The HPLC system consisted of a Model L-7100 pump, a Model L-7200 auto-sampler and a Model L-7480 fluorescence detector, all supplied by Hitachi (Tokyo, Japan). A Hypersil ODS column (5 μm, 25 cm×4.6 mm I.D.) was used as the analytical column. The temperature of the column was maintained at 40 °C using a Bio-Rad Column Oven (Hercules, CA, USA). Chromatograms were recorded and processed by an SISC Data Station (Scientific Information Service Corporation, Taipei, Taiwan). The chromatographic analysis was performed in an air-conditioned laboratory at 20 °C. Separations were conducted using a mobile phase consisted of acetonitrile-50 m*M* sodium dihydrogen phosphate, pH 4.6 (60:40, v/v) and pumped at a flow-rate of 1 ml/min. The prepared mobile phase was filtered through a 0.45-μm pore-size membrane filter and ultrasonically degassed prior to use. The eluants were detected by fluorescence at an excitation wavelength of 295 nm and an emission wavelength of 425 nm, and the peak area recorded.

2.3. Standards and controls

Stock solutions of delavirdine (2 mg/ml in methanol) and internal standard cisapride (1 mg/ml in acetonitrile) were prepared monthly and kept tightly sealed at −80 °C. The stock solution of delavirdine was diluted with pooled drug-free plasma to give the calibration standards at concentrations of 25, 50, 100, 250, 500, 1000, 2500, 5000, 12 500 and 25 000 ng/ml. The quality controls were prepared independently at concentrations of 25, 200, 2000 and 20 000 ng/ml and stored at −80 °C until used. Working solution of cisapride was obtained by diluting the stock solution in acetonitrile to 10 μg/ml.

2.4. Sample preparation

The samples to be analyzed were removed from the freezer and thawed. Calibration standards, quality controls, and study samples were pipetted into 1.5 ml microcentrifuge tubes and processed as a batch. To 50 μl of plasma samples were added 150 μl of the internal standard working solution. After vortex-mixed for 30 s and upon centrifugation at 15 850 *g* for 10 min, an aliquot of the supernatant was injected onto the column for HPLC analysis.

2.5. Calibration and validation

The model for the calibration curve of delavirdine used a natural logarithmic transformation of the peak area ratio of delavirdine to cisapride (PAR) and the delavirdine concentration (*C*), as given in the following equation: $\ln(\mathrm{PAR}) = \mathrm{Slope} * \ln(C) + (y \text{ intercept})$. The slope and *y* intercept were determined by linear regression analysis (Excel 97 SR-1, Microsoft Corp., Redmond, WA) using nominal concentrations and measured PARs from calibration standards. Unknown delavirdine concentrations were estimated by inverse prediction from the calibration equation. A complete calibration curve was generated with each analytical run.

Within-day assay precision was determined by analyzing the four spiked controls six times over 1 day in random order, while between-day precision was determined from the analysis of each control once on each of six different days. Assay precision was assessed by expressing the standard deviation of the measurements as a percentage of the mean value (coefficient of variation, C.V.). Accuracy was measured as the percent deviation from the nominal concentration for each spiked controls (relative error, %E). The lower limit of quantitation (LOQ) was the lowest non-zero concentration level, which could be accurately (%E <20%) and reproducibly (C.V. <20%) quantitated [12]. The selectivity of the assay was examined in relation to interference from endogenous substances in drug-free plasma.

Blank plasma were spiked with 25, 1000, 25 000 ng/ml of delavirdine and the plasma samples were stored at 4 °C. The concentration of delavirdine after 7 days storage period was compared to the initial concentration as determined for the samples that were freshly prepared and processed immediately. The stability of delavirdine in processed samples left at ambient temperature (ca. 20 °C) in autosampler vials was followed for 24 h.

2.6. Pharmacokinetic application

The assay was applied to a single dose (0.18 mg delavirdine mesylate) pharmacokinetic study in rats. Male Sprague–Dawley rats were obtained from the Animal Breeding Center of National Cheng Kung University. The study protocol complied with the Institutional Guidelines on Animal Experimentation of National Cheng Kung University. After intravenous bolus administration, blood samples for analytical determinations were collected at specific time intervals for 120 min. Plasma samples were stored at −80 °C until analysis. The pharmacokinetic parameters of delavirdine were determined by compartmental analysis. Nonlinear regression modeling was used to fit the measured delavirdine plasma concentrations to a one-compartment model with first-order elimination. The primary pharmacokinetic parameters (clearance, volume of distribution and terminal half-life) were estimated using the software *WinNonlin Pro 2.0* (Pharsight Inc., Mountain View, CA, USA).

3. Results and discussion

3.1. Chromatography

Fig. 2A shows a chromatogram of a pooled blank plasma fortified with 25 ng/ml delavirdine and the internal standard. Fig. 2B,C,D represent chromatograms of extracts from pre-dose, 10 and 120 min after administration of 0.18 mg delavirdine mesylate to a rat, respectively. The concentration of delavirdine were 261 and 41.8 ng/ml, respectively, for Fig. 2C,D. Delavirdine and cisapride were eluted as sharp and symmetrical peaks after 5.3 and 6.5 min, respectively. The total run time was less than 8 min. As can be seen in Fig. 2, a good separation of delavirdine and the internal standard was achieved under the chromatographic conditions specified in Section 2.

The simple protein precipitation was sufficient to isolate the analytes from the biological matrix without any interfering endogenous peaks. The method is specific for delavirdine. No interfering peaks were observed at the retention of delavirdine when blank pre-dose rat plasma samples were analyzed (Fig. 2B). Based on our previous experience gained with cyano column [4], and the experience of others with YMC 5-μm basic [5] and cyano, Phenyl and C_8 columns [9], the capacity factor of desisopropyl-delavirdine should be very small under the present chromatographic conditions. It probably eluted among the solvent peaks, therefore did not interfere with delavirdine.

3.2. Calibration and validation

The linearity of assay was evaluated by analysis of calibration standards over the range of 25–25 000 ng/ml of delavirdine. The calibration curves were linear over the range with coefficients of regression being typically greater than 0.999. Linear regression analysis yielded mean (±SD, $n=6$) values for slope and intercept for the equation of the calibration curve, using 50 μl plasma samples, of $\ln(\mathrm{PAR}) = (0.986 \pm 0.005)^* \ln C + (-5.89 \pm 0.05)$.

Precision and accuracy (25–20 000 ng/ml) were investigated by replicated analyses of spiked controls (Table 1), and in all cases the within-day and between-day precision was acceptable at a C.V. of 9.3% or less. In addition, accuracy was within 9.2% when compared with nominal concentrations across this range. For this validation the LOQ was set at 25

Table 1
Within-day and between-day accuracy and precision for the determination of delavirdine in 50 μl plasma ($n=6$)

Within-day				Between-day		
$C_{nominal}$ (ng/ml)	C_{est} (ng/ml)	C.V. (%)	Error (%)	C_{est} (ng/ml)	C.V. (%)	Error (%)
25	27.3	4.5	9.2	25.9	8.2	3.7
200	200	3.3	0.2	204	5.8	2.0
2000	1988	3.7	−0.6	1988	5.8	−0.6
20 000	20 600	2.5	3.0	19 829	9.3	−0.9

$C_{nominal}$: Nominal concentration; C_{est}: Estimated concentration.

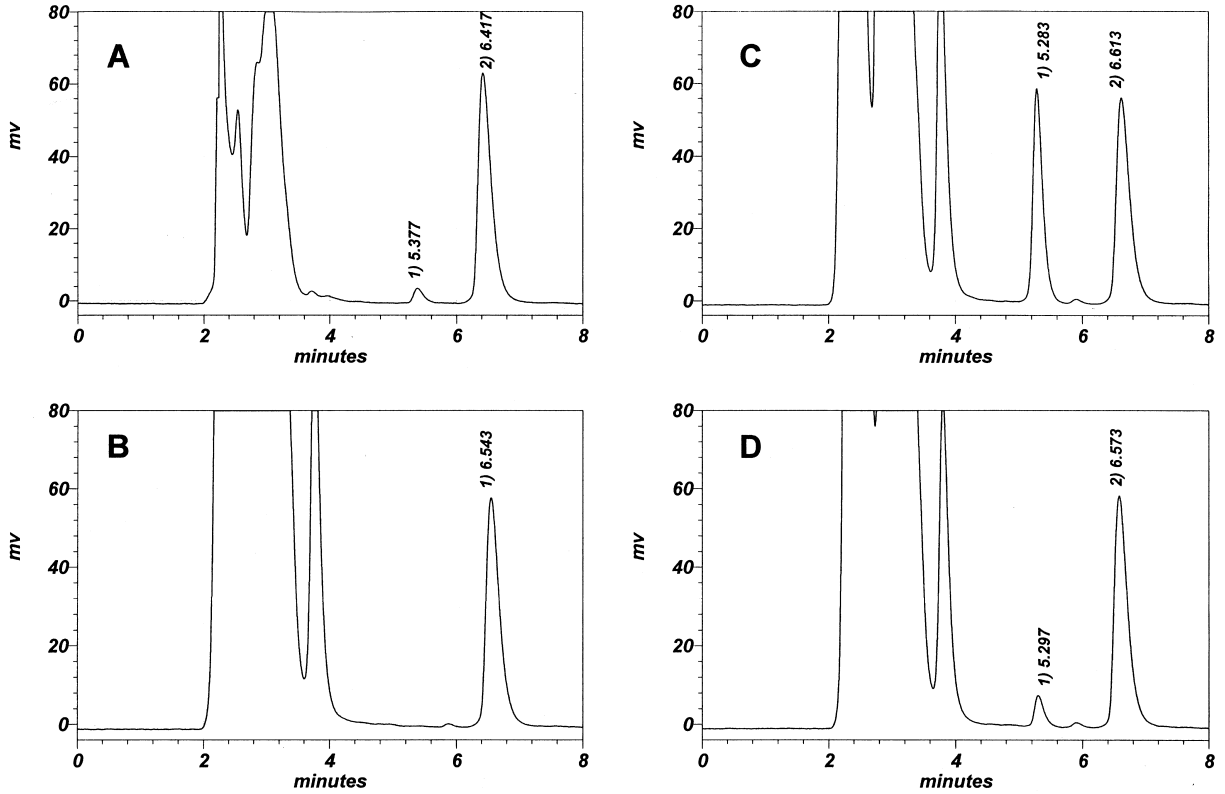


Fig. 2. HPLC chromatograms of a pooled blank plasma sample spiked with 25 ng/ml of delavirdine (A), pre-dosing (B), 10 min (C) and 120 min (D) plasma samples from a rat after intravenous bolus of 0.18 mg delavirdine mesylate.

ng/ml. This is similar to values previously reported at 25 [4,10] and 50 [9] ng/ml in 200 μl of plasma using fluorescence detection, and is much sensitive than using UV detection. The LOQ for UV detection at 260 and 265 nm using 0.5 ml plasma/serum were 75 and 110 ng/ml, respectively [7,8], and that for 254 nm using 1 ml serum was 100 ng/ml [6]. Results of the stability studies indicated that delavirdine is stable for a minimum of 24 h at ambient temperature in processed samples, 7 days at 4 °C in plasma (Table 2), which were in agreement with previous studies [9,11].

3.3. Pharmacokinetic applicability

The assay was applied to a preliminary pharmacokinetic experiment in rats. A single bolus dose of 0.18 mg of delavirdine mesylate was administered

Table 2
Stability of delavirdine in spiked human plasma samples (Mean±SD, $n=6$)

Concentration (ng/ml)	Percentage as the initial concentration remained	
	Processed, 24 h, 20 °C	7 days, 4 °C
25	106.0±9.1	101.4±2.4
1000	99.3±1.2	94.2±0.7
25 000	100.7±7.6	99.8±0.2

intravenously to three male rats. Blood samples were collected at scheduled intervals. The mean (±SD) plasma concentration-time profile is illustrated in Fig. 3. The pharmacokinetic parameter estimates (mean±SD) of clearance, volume of distribution and terminal elimination half-life were 23±3 ml/min/kg, 1.3±0.2 l/kg and 37±2 min, respectively. These indicated that delavirdine distributed widely into tissues and was cleared rapidly from the body. The results showed that this simple and rapid method is sufficiently sensitive to follow blood level of delavirdine for more than three elimination half-lives after dosing, which is very important in obtaining accurate estimates for the pharmacokinetic parameters.

3.4. Method development

The reported HPLC assays for delavirdine in the literature have employed various type of columns for separation, such as cyano [4,9,10], phenyl [9], C_8 [6,9] and C_{18} [7,8,11]. The mobile phase is delivered under isocratic [7,11] or gradient conditions [4,6,8–10], with the pH value of the buffer solution/mobile phase varies from 2.7 to 7.5. In view of the simplicity and the stability of the column, we have used a pH of 4.6 buffer solution for mobile phase in isocratic mode. Among the reported assays, those used fluorescence detection [4,9–11] were much sensitive (LOQ of 10–50 ng/ml with 200 μl plasma) as compared to those used UV detection (LOQ of 75–110 ng/ml with 500–1000 μl plasma/serum) [6–8]. Thus, fluorescence detection was employed in this study.

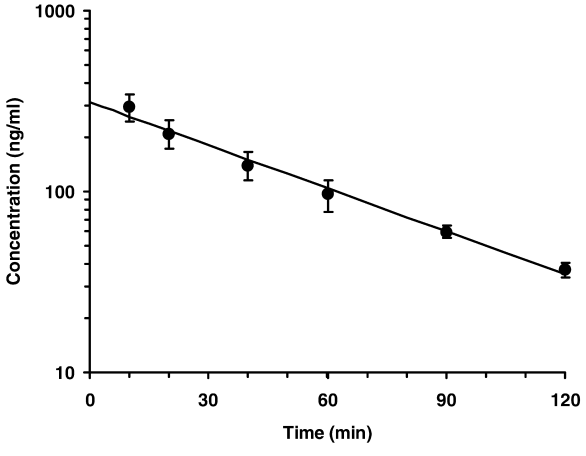


Fig. 3. The mean (±SD) plasma concentration-time profile of delavirdine after intravenous bolus of 0.18 mg delavirdine mesylate to male rats (n=3). The solid line represents fitted plasma concentrations according to a one-compartment model using *WinNonlin Pro 2.0*, Pharsight Co., USA.

We had used a method based on Staton's to study the pharmacokinetics of delavirdine and its desalkyl metabolite in AIDS patients [4,10]. In our previous study the internal standards used were atevirdine and desalkyl atevirdine [4,10], whereas that of Staton's was U-88822 (a pyridineamine) [9]. These compounds all have native fluorescence, however, none of them were readily available. Hence, in a recent HPLC assay for delavirdine with fluorescence detection, no internal standard was used [11]. In the present study, cisapride was chosen as the internal standard because it is commercially available and its peak was sufficiently separated from that of delavirdine, and most importantly it also has native fluorescence. Like delavirdine, cisapride is lipophilic, highly bound to plasma protein and is also a CYP3A4 substrate.

The method of Staton's was validated over the range of 10–7915 ng/ml for delavirdine, and was applied to characterize the plasma levels in a subject who received a single oral dose of 300 mg delavirdine mesylate [9]. On considering the steady-state peak concentration of delavirdine in clinical practice [1,2,4], we have extended the dynamic calibration range in the present assay.

Because of the clinical status of HIV-infected patients, particular of the very young children, it is often difficult to obtain suitable amount of blood from these patients. Therefore, in this study efforts were made to reduce substantially the amount of plasma sample needed, from 200 to 50 μl, for the quantitation of delavirdine. This is most useful in reducing the blood collection, offering the possibility to make sufficient numbers of blood samples for pharmacokinetic study, and minimizing the amount of blood-derived biological waste that need to be disposed with care.

4. Conclusion

In conclusion, a new HPLC method for the determination of delavirdine has been developed. The method offers the sensitivity and selectivity for

monitoring therapeutic concentration of delavirdine. The method is flexible and required only 50 μl plasma, making it suitable for studying the pharmacokinetics of delavirdine in HIV patients, children, and small animals. The application of this method was demonstrated in a pharmacokinetic study in rats.

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