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Simple, rapid and sensitive high-performance liquid chromatographic determination of delavirdine and its N-desisopropyl metabolite in human plasma

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Abstract

A method for the determination of a bisheteroarylpiperazine, non-nucleoside HIV-1 reverse transcriptase inhibitor, delavirdine, and its N-desisopropyl metabolite in human plasma, is described. Samples were deproteinized by addition of two parts of a solution of internal standard in acetonitrile (1 μg/ml) to one part plasma. The supernatant was diluted with 10 m*M* phosphate buffer, pH 6.0, and injected onto the HPLC system. Fluorescence of the eluent was monitored with excitation at 302 nm and emission at 425 nm. Quantitation of delavirdine and its metabolite was achieved by comparing the peak-height ratio of each component relative to the internal standard to a through-the-origin linear regression curve determined from fortified plasma calibration standards. The assay was linear over the concentration range 0.02–17 μM for both delavirdine and its metabolite. The precision of the method, as expressed by the mean C.V. of the back-calculated, non-zero, standard concentrations, was ± 4.4% for delavirdine and ± 4.3% for the metabolite. The assay has been validated and utilized to analyze samples from human and animal pharmacokinetic studies.

1. Introduction

Delavirdine mesylate (Fig. 1) is one of a group of bisheteroarylpiperazine compounds (BHAPs) being developed by The Upjohn Company for the treatment of acquired immunodeficiency syndrome (AIDS). In vitro testing has demonstrated that delavirdine is a potent and selective non-nucleoside inhibitor of the reverse transcriptase of type 1 human immunodeficiency virus (HIV-1) [1,2], the causative agent of AIDS. Furthermore, BHAP resistant mutants of HIV-1

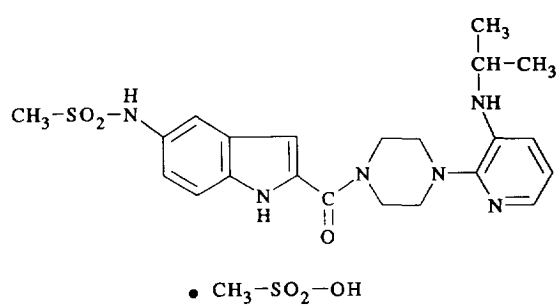

Fig. 1. Delavirdine mesylate (M_r = 552.68)

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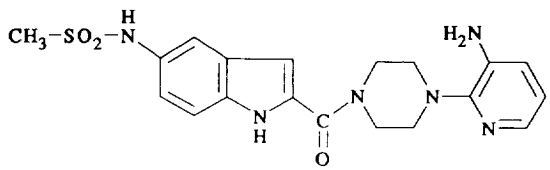

Fig. 2. Metabolite, U-96183 (M_r = 414.49)

appear to have increased sensitivity to other non-nucleoside inhibitors [3] making delavirdine a candidate for combination therapy [4]. Delavirdine mesylate is currently in phase III clinical trials.

Preclinical studies demonstrated that the pharmacokinetics of delavirdine were non-linear [5] and, consequently, dose escalations in the safety and tolerance clinical trials were plasma-concentration controlled [6]. Results were needed within 24 h and a rapid method to quantitate delavirdine and its N-desisopropyl metabolite, U-96183 (Fig. 2), in human plasma was required.

A quantitative HPLC method employing the deproteinization of human plasma samples with an acetonitrile solution of a delavirdine analog, U-88822 (Fig. 3), as an internal standard is presented here.

2. Experimental

2.1. Materials

Delavirdine mesylate, 1-[3-[(1-methylethyl)-amino] - 2 - pyridinyl] - 4 - [[5 - [(methylsulfonyl)-amino] - 1H - indol - 2 - yl]carbonyl] - piperazine, the N-desisopropyl metabolite of delavirdine, U-96183, 1 - (3 - amino - 2 - pyridinyl) - 4 - [[5-[(methylsulfonyl)amino]-1H-indol-2-yl]carbonyl] - piperazine, and the internal standard U-88822, 2 - [4 - (5 - hydroxy - 1H - indol - 2 - ylcarbonyl)-1-piperazinyl]-N-(1-methylethyl)-3-pyridineamine were prepared by The Upjohn Company (Kalamazoo, MI, USA). Acetonitrile and methanol were UV grade from Burdick and Jackson (Muskegon, MI, USA). All water was type I obtained from a Milli-Q system from Millipore (Bedford, MA, USA). The potassium phosphate monobasic from Mallinckrodt (Paris, KY, USA) and the potassium hydroxide 45% from J.T. Baker (Philipsburg, NJ, USA) were analytical reagent grade. Blank human plasma from individual volunteers was obtained from a plasmaphoresis protocol at The Jasper Clinical Investigational Unit, The Upjohn Company (Kalamazoo, MI, USA). Methanolic working standards were stored in screw-capped 2-ml polypropylene test tubes from Sarstedt (Newton, NC, USA). Quality control and subject samples were stored in screw-capped 16 × 79 mm polypropylene sample tubes from Sarstedt. Sample aliquots were processed in round-bottom 13 × 100 mm polypropylene extraction tubes from Baxter Scientific (McGaw Park, IL, USA). Supernatant was transferred to 1-ml polypropylene HPLC vials from Sun Brokers (Wilmington, NC, USA) and the vials were sealed with teflon-coated septa crimp caps, also from Sun Brokers.

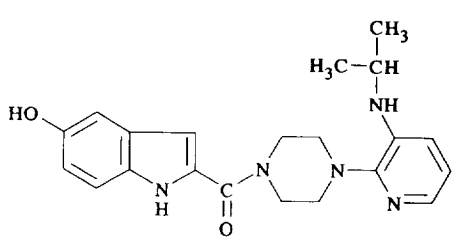

Fig. 3. Internal standard, U-88822 (M_r = 379.47)

2.2. Reagents

A solution of 5% potassium hydroxide was prepared by dilution of potassium hydroxide 45% AR with type I water. The 5% potassium hydroxide was used to adjust the pH of a 10 m*M* potassium phosphate monobasic solution to 6.0. Mobile phase was prepared by mixing 10 m*M* potassium phosphate buffer, pH 6.0, with acetonitrile (2:1), filtering the mixture through a 0.4-μm polycarbonate Nuclepore filter from Anspec (Ann Arbor, MI, USA) and degassing it with a sparge of high purity helium at 3 psi for 10 min. A 1 μg/ml working solution of the internal standard, U-88822, was prepared in acetonitrile.

2.3. Instrumentation

The high-performance liquid chromatographic system (HPLC) used a Waters Model 590 programmable solvent delivery module (Milford, MA, USA), pumping the mobile phase at 1.5 ml/min. A 25-μl aliquot of sample was introduced into the system via a Hewlett-Packard Model 1050 autosampler (Naperville, IL, USA). The tray of the autosampler was maintained at a temperature of 1–8°C by a NESLAB RTE-100 refrigerated bath/circulator (Newington, NH, USA) using a mixture of 50% ethylene glycol from Mallinckrodt (Paris, KY, USA) and 50% water. The chromatographic separation was effected on a Zorbax SB CN, 150 × 4.6 mm I.D., 5 μm analytical column from Mac-Mod Analytical (Chadds Ford, PA, USA) along with a RP-CN NEWGUARD, 15 × 3.2 mm I.D., 7 μm precolumn from Applied Biosystems (Foster City, CA, USA). The column effluent was monitored using a Waters Model 470 scanning fluorescence detector operated with 18 nm slit widths at 302 nm excitation and 425 nm emission. The detector output was monitored with a Hewlett-Packard Model 3395 integrator and a Harris NightHawk 4400 superminicomputer (Fort Lauderdale, FL, USA) via a Perkin-Elmer Series 900 interface (Norwalk, CT, USA). Results were calculated on the NightHawk computer using an in-house developed chromatography system.

A Thermo-Lyne Dri Bath from VWR (Baltimore, MD, USA) was used to heat samples for the deactivation of HIV-1 samples.

2.4. Sample preparation

A calibration curve covering the range 0.02–17 μ*M* for both delavirdine and the metabolite was determined in duplicate from drug-fortified plasma calibration standards. Two standard stock solutions (A and B) were prepared in acetonitrile–methanol (1:9). The concentrations of delavirdine mesylate and the metabolite for stock A were approximately 90 and 120 μg/ml, respectively, and for stock B were 220 and 310 μg/ml, respectively. A set of seven methanolic working standards was prepared from dilutions of each stock solution (0.1 ml/100 ml, 2 ml/250 ml, 2 ml/25 ml, and 4 ml/5 ml for A; 0.1 ml/100 ml, 1 ml/100 ml, and 1 ml/10 ml for B). Each methanolic working standard was subdivided into 200-μl aliquots in screw-capped 2-ml polypropylene test tubes and stored at −20°C. For each assay, a set of methanolic working standards was warmed to room temperature prior to use and calibration standards were prepared by adding 20 μl of each methanolic working standard to separate 200-μl aliquots of blank human plasma.

Three quality control samples containing both delavirdine and the metabolite were prepared in human plasma at concentrations such that the low control (0.075 μ*M*) was above the two lowest standards, the medium control (0.75 μ*M*) fell between the middle standards, and the high control (15 μ*M*) was below the highest standard for each compound. A quality control stock solution of approximate concentration 100 μg/ml for delavirdine mesylate and 150 μg/ml for the metabolite was prepared in methanol. A set of three intermediate methanolic control solutions was prepared from dilutions of the stock solution: 4 ml/10 ml for the high control, 2 ml/100 ml for the medium control which was subsequently diluted 1 ml/10 ml for the low control. Quality control samples in human plasma were prepared by transferring 1 ml of each intermediate methanolic control solution directly into 50 μl of propylene glycol in separate 10-ml volumetric flasks. The methanol was evaporated under a stream of filtered nitrogen at ambient temperature and each flask was brought to volume with blank human plasma. Evaporation of the methanol control solution in the absence of propylene glycol resulted in poor recovery of the metabolite. Each quality control sample was subdivided into 1-ml aliquots in screw-capped 16 × 79 mm polypropylene sample tubes and stored at −20°C.

The internal standard working solution was prepared by dissolving approximately 1.0 mg of U-88822 in 100 ml of acetonitrile and subsequently diluting 10 ml of this solution to 100 ml with acetonitrile.

A 200-μl aliquot of plasma (quality control sample, subject sample or blank for the prepara-

tion of calibration standards) was transferred to a polypropylene extraction tube. Calibration standards were prepared in duplicate by addition of 20 μl of each methanolic working standard to two separate aliquots of blank human plasma. Every ten subject samples were bracketed by a quality control sample. A 400-μl aliquot of internal standard working solution was added to each tube. All tubes were vortex-mixed for 1 min and centrifuged for 5 min with a minimum relative centrifugal force (RCF) of 750 in order to pellet the precipitated proteins. A 200-μl aliquot of supernatant was transferred to a 1-ml polypropylene HPLC vial. A 400-μl aliquot of 10 m*M* potassium phosphate buffer, pH 6.0, was added to each vial. The vials were crimp-capped, vortex-mixed for 30 s and transferred to the refrigerated tray of the HPLC autosampler.

The chromatographic system was equilibrated with fresh mobile phase for 30–45 min. Typical injection intervals were about 11 min at a flow-rate of 1.5 ml/min. Retention times were approximately 3 min for the metabolite, 7.5 min for the internal standard and 9 min for delavirdine. Typical system performance was approximately 10 000 plates/m for the metabolite, 19 000 plates/m for the internal standard and 31 000 plates/m for delavirdine with a minimum resolution of 1.7 between the internal standard and delavirdine. After each run (approximately 130 samples), the analytical column was flushed with acetonitrile–type I water (1:2) at a flow-rate of 1 ml/min for one hour. A new guard column was installed prior to each run.

Quantitation of delavirdine and the metabolite was achieved by calculating the peak-height ratio of each component relative to the internal standard, and comparing the ratio to an unweighted, through-the-origin linear regression curve determined from the fortified plasma calibration standards.

The stabilities of delavirdine and the metabolite were determined in human plasma stored at room temperature (21–23°C), 4°C and −20°C for various time intervals. The procedure measured the concentration of delavirdine and the metabolite in both stored and freshly prepared samples on the same day.

The stability of prepared samples stored in a refrigerated autosampler tray at 1–8°C was investigated. Sample preparation was scaled up proportionately and split equally into HPLC vials to achieve 16 identical prepared samples for each quality control concentration level. All samples were placed in the refrigerated tray and a different sample was injected every hour over a 48-h period.

The stability of delavirdine and the metabolite in human plasma heated at 56°C for 30 min to deactivate the reverse transcriptase activity of HIV-1 [7] was studied. Quality control samples in capped polypropylene sample tubes were heated at 56°C for 15, 30 and 60 min intervals and then frozen at −20°C. This procedure was repeated at 51°C and again at 61°C to ascertain stability over this temperature range. All samples, along with a set of untreated quality control samples, were assayed in duplicate.

Drugs commonly co-administered in HIV-1 individuals were evaluated as possible chromatographic interferences (Table 1). Individual solutions of approximately 10 μg/ml were prepared in methanol–acetonitrile (1:9). Since the flucytosine solution was prepared from a dosage formulation whose ratio of drug to excipients was unknown, the sample size was increased approximately four times. A 200-μl aliquot of each solution and a 400-μl aliquot of 10 m*M* phosphate buffer, pH 6.0, were transferred to a 1-ml polypropylene HPLC vial. The vials were crimp-capped, vortex-mixed for 30 s and introduced onto the HPLC system. Since there was no initial 1:2 dilution inherent in the plasma deproteinization step, the theoretical plasma concentrations for all compounds except for flucytosine were back-calculated to be approximately 30 μg/ml. Chromatographic responses within ± 0.5 min retention windows for delavirdine, the metabolite or the internal standard were quantitated against single point calibrations for each analyte in order to estimate the level of interference.

3. Results

Selection of a CN column was based upon comparison of elution characteristics of the com-

Table 1
Compounds possibly co-administered in HIV-1 subjects evaluated for chromatographic interferences

Compound	Theoretical plasma Concentration (μg/ml)	Retention time (min)
Acetaminophen	28.8	2.0
Acetylsalicylic acid	31.2	1.6
Amikacin	33.9	none
Amphoteracin B	31.8	2.9, 3.6[a]
Caffeine	33.6	2.9
Ciprofloxacin	30.6	4.1
Clindamycin	31.5	none
Dapsone	31.8	3.6[a], 5.1
Dilantin	35.7	none
Flucytosine	116	1.6
Ibuprofen	31.2	none
Isoniazid	38.4	none
Loperamide	27.6	none
Nicotine	38.4	none
Pentamidine isethionate	33.0	none
Primaquine	30.3	none
Pyrimethamine	32.1	7.0
Rifampin	39.6	none
Sulfamethoxazole	38.7	2.7
Trimethoprim	30.6	3.4[a]
AZT (zidovudine)	30.9	none

[a] Possible interference with metabolite, U-96183.

pounds of interest on three different Zorbax Stable Bond reverse phase packings: CN, Phenyl and C_8. The metabolite was retained consistently between columns, but the internal standard and delavirdine were retained approximately twice as long on both the Phenyl and C_8 columns relative to the CN column (Fig. 4). The Phenyl and CN columns also demonstrated separation of the metabolite from two potential matrix interference peaks while the first of these peaks co-eluted with the metabolite on the C_8 column (Fig. 4). Therefore, the CN column was chosen to enhance selectivity and minimize the run time.

The effect of the buffer pH on the retention of the compounds extracted from plasma was also investigated on a CN column. The capacity factors of all three analytes increased as the pH was raised from 2.9 to 4.8 and then began to decrease as the pH was raised further (Fig. 5). The capacity factors of the two potential matrix interferences decreased steadily as the pH was

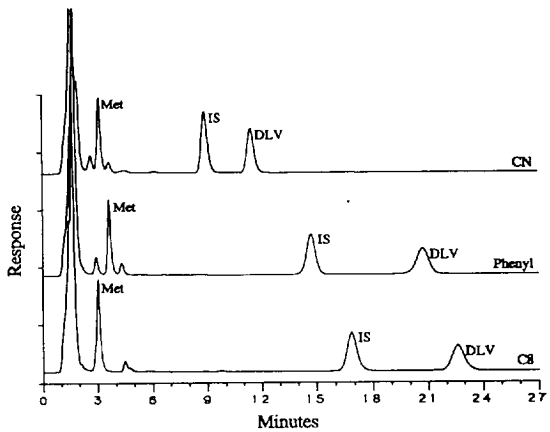

Fig. 4. Comparison of stationary phases. Met = metabolite; IS = internal standard; DLV = delavirdine.

raised from 2.9 to 6.0, at which point they were no longer retained nor interfered. Therefore, a pH of 6.0 for the 10 m*M* potassium phosphate buffer was used to eliminate potential interferences with the metabolite from these matrix components.

This method was successfully used to assay samples from an escalating single dose clinical protocol over a six month period in which delavirdine mesylate was administered orally to healthy volunteers. Chromatography of predose and C_{max} samples from a subject who received 300 mg of delavirdine mesylate shows the metab-

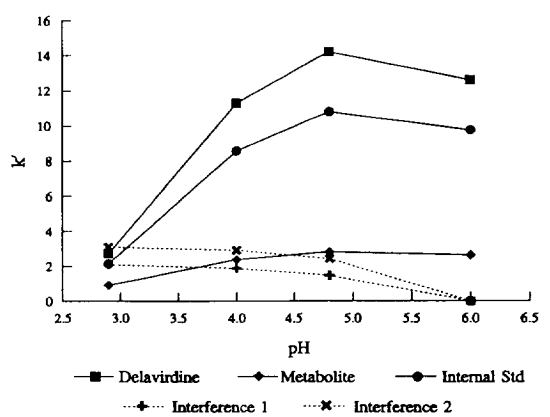

Fig. 5. Effect of pH upon elution of compounds of interest. Mobile phase: acetonitrile–60 m*M* acetic acid (28:72) adjusted to pH with NH_4OH. Column: Zorbax SB CN, 80 × 4 mm I.D., 5 μm.

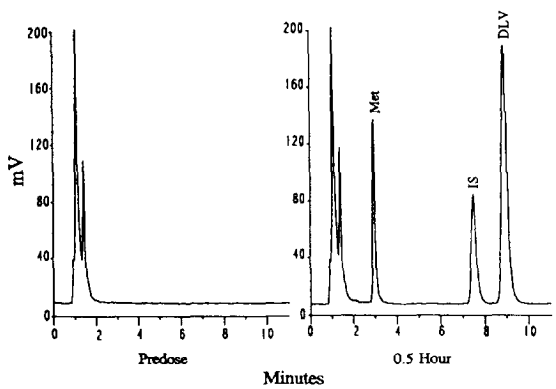

Fig. 6. Representative chromatography from a human subject who received a single oral dose of 300 mg delavirdine mesylate (plasma concentrations of DLV and Met at 0.5 h were 9 and 2.5 μM, respectively). Met = metabolite; IS = internal standard; DLV = delavirdine.

olite eluting at about 3 min, the internal standard at about 7.5 min and the drug at about 9 min (Fig. 6). A representative concentration–time profile for the subject is shown in Fig. 7.

Calibration curves for both delavirdine and the metabolite were linear over the range of 0.02 to 17 μM. The precision of the method, as expressed by the mean C.V. of the back-calculated, non-zero, standard concentrations, was +/− 4.4% for delavirdine and +/− 4.3% for the metabolite. Inter-day variability, as demonstrated by the inter-assay C.V. for each quality control concentration, did not exceed 6.4% (Table 2). Intra-day variability, as demonstrated by the intra-assay C.V. for each quality control concentration, did not exceed 8.7%. The lower limit of quantitation (LOQ), as determined by the lowest detected standard concentration where the mean back-calculated concentration was within ± 20% of theory and the inter-assay C.V. did not exceed 20%, was 0.0216 μM for both delavirdine and the metabolite.

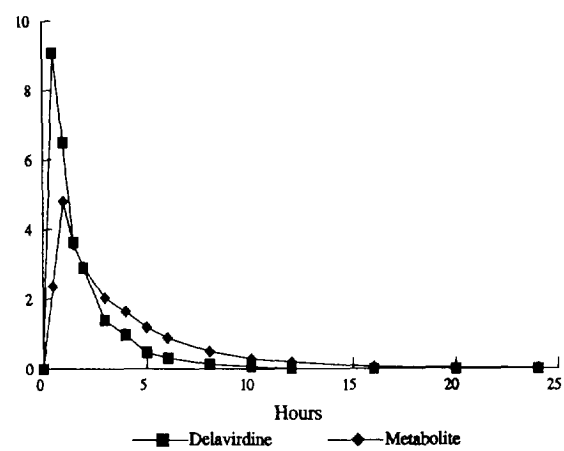

Fig. 7. Plasma concentration–time profile for a human subject who received a single oral dose of 300 mg delavirdine mesylate.

Table 2
Inter-day accuracy and precision as demonstrated by quality control sample recoveries

Concentration (μM)	n	Mean (μM)	C.V. (%)
Delavirdine			
0.0747	43	0.0719	6.4
0.7469	43	0.7062	4.4
14.94	43	14.11	3.5
Metabolite			
0.0743	43	0.0718	6.2
0.7426	43	0.6925	3.7
14.85	41	14.05	3.3

Results of the stability in plasma studies indicated that delavirdine is stable for a minimum of 3 days at ambient temperature (21–23°C), 7 days at 4°C, and 1 year at −20°C (Table 3). The metabolite demonstrated stability for a minimum of 3 days at ambient temperature (21–23°C), 7 days at 4°C, and 89 days at −20°C (Table 3). Prepared samples stored in a refrigerated autosampler tray at 1–8°C exhibited no decomposition of delavirdine, the metabolite or the internal standard over a 48-h period. Results of the heat deactivation stability study indicated that no significant decomposition occurred when samples were treated at temperatures up to 61°C for 60 min (Table 4).

No chromatographic interferences with either the internal standard or delavirdine were observed for the compounds commonly administered to HIV-1 subjects listed in Table 1. Three of the compounds, amphoteracin B, dapsone, and trimethoprim, exhibited potential chromatographic interferences with the metabolite. Peak

Table 3
Comparison of fresh vs. stressed control recoveries for the determination of stability of delavirdine and metabolite in human plasma under different storage conditions

Temperature (°C)	Day		Recovery (mean ± S.D., $n = 4$) (%)	
			Delavirdine	Metabolite
Ambient	3	Fresh	96.14 ± 2.53	97.45 ± 2.92
		Stressed	100.3 ± 2.1	101.6 ± 2.0
4	7	Fresh	104.4 ± 11.3	105.1 ± 9.5
		Stressed	104.6 ± 4.0	107.0 ± 5.7
−20	371	Fresh	91.27 ± 2.05	106.7 ± 2.4[a]
		Stressed	90.93 ± 3.41	96.20 ± 1.94[a]

[a] Day 89.

Table 4
Recoveries of delavirdine and its N-desisopropyl metabolite from quality control samples heat-deactivated at 61°C

Compound	Recovery (mean ± S.D., $n = 6$) (%)		
	Control	30 Min	60 Min
Delavirdine	100.0 ± 3.6	105.7 ± 4.4	104.9 ± 3.2
Metabolite	100.0 ± 0.8	101.2 ± 3.5	101.8 ± 2.3

plasma concentrations of amphoteracin B, 2.0 μg/ml [8], would produce a peak roughly equivalent to 0.008 μM of the metabolite which is below the LOQ. Peak plasma concentrations of dapsone, 7.0 μg/ml [9], would produce a peak roughly equivalent to 0.067 μM of the metabolite. Peak plasma concentrations of trimethoprim, 8.8 μg/ml [10], would produce a peak roughly equivalent to 0.117 μM of the metabolite.

The utility of the method has been demonstrated by the diversity of studies for which it has since been employed. These include multiple dose pharmacokinetic studies in both healthy and asymptomatic HIV-1 subjects as well as toxicokinetic monitoring in rats, dogs, rabbits, monkeys and micropigs. The short analysis time enabled the rapid turnaround of results and subsequent dose adjustments to achieve targeted plasma concentrations. In addition, the simplicity of the sample preparation facilitated the automation of the method on a Zymark robot.

The method described in this report has been shown to be precise and sensitive enough for use in pharmacokinetic studies where the minimum quantifiable level required is 0.02 μM or greater. Furthermore, the brevity of sample analysis was conducive to rapid therapeutic drug monitoring.

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