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Journal of Chromatography B, 727 (1999) 151–157

JOURNAL OF CHROMATOGRAPHY B

Rapid quantification of delavirdine, a novel non-nucleoside reverse transcriptase inhibitor, in human plasma using isocratic reversed-phase high-performance liquid chromatography with fluorescence detection

Agnes I. Veldkamp[a,*], Rolf P.G. van Heeswijk[a], Richard M.W. Hoetelmans[a], Pieter L. Meenhorst[b], Jan W. Mulder[b], Joep M.A. Lange[c], Jos H. Beijnen[a]

[a]*Department of Pharmacy and Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
[b]*Department of Internal Medicine, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
[c]*National AIDS Therapy Evaluation Centre, Department of Internal Medicine, Academic Medical Centre, Amsterdam, The Netherlands*

Received 23 November 1998; received in revised form 17 February 1999; accepted 17 February 1999

Abstract

Delavirdine is a novel non-nucleoside reverse transcriptase inhibitor for the treatment of HIV-1-infected patients. A simple and rapid high-performance liquid chromatographic method for the quantification of delavirdine in human plasma suitable for drug monitoring in patients is described. Sample pretreatment consists of protein precipitation with acetonitrile and subsequent evaporation of the extract to concentrate the analyte. The drug is separated from endogenous compounds by isocratic reversed-phase, high-performance liquid chromatography coupled with fluorescence detection. The optimal excitation and emission wavelengths are 300 and 425 nm, respectively. The method has been validated over the range of 50–50 000 ng/ml using only 200 μl of plasma samples. The assay is linear over this concentration range as indicated by the *F*-test for lack of fit. Within- and between-day precisions are less than 4.4% for all quality control samples. The lower limit of quantitation is 50 ng/ml. Recovery of delavirdine from human plasma is 93.8%. Delavirdine is stable under various conditions, for example 1 h at 60°C and one week at 4°C. This validated assay is suited for use in pharmacokinetic studies with delavirdine and can readily be implemented in the setting of a hospital laboratory for the monitoring of delavirdine concentrations.

Keywords: Delavirdine

1. Introduction

Delavirdine (U90152, Rescriptor, Fig. 1) belongs to a new class of antiretroviral drugs: the non-nucleoside reverse transcriptase inhibitors. It is a bis(heteroaryl)piperazine-derivative. Delavirdine is a noncompetitive inhibitor of the viral replicative enzyme reverse transcriptase, an important therapeutic target in the treatment of human immunodeficiency virus infection (HIV-1). The drug is inactive against HIV-2 reverse transcriptase and does not bind to human DNA polymerases [1].

*Corresponding author. Tel.: +31-20-512-4731; fax: +31-20-512-4753.
E-mail address: apabg@slz.nl (A.I. Veldkamp)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00081-X

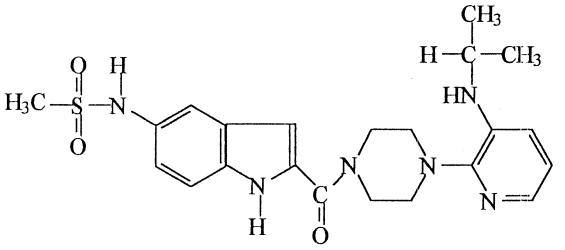

Fig. 1. Molecular structure of delavirdine.

Treatment of patients with delavirdine monotherapy rapidly selects for resistant isolates of HIV-1, the most common mutation is K103N of the reverse transcriptase enzyme [2,3]. However, when given in combination with nucleoside reverse transcriptase inhibitors and protease inhibitors, delavirdine has demonstrated a potent antiviral effect and is therefore an important addition to the current antiretroviral drug armamentarium [1,2,4,5].

Delavirdine is metabolized by the cytochrome P450 isoenzyme 3A4 (CYP3A4). It reduces the enzyme activity and thereby inhibits its own metabolism. This results in non-linear pharmacokinetics, with values for the area under the plasma concentration versus time curve (AUC), peak and trough concentrations increasing disproportionately with increasing dose [6].

Delavirdine is a very weak base and practically insoluble at pH>3. No water-soluble salt form is available. Delavirdine should not be administered with didanosine (formulation contains additives to increase pH of the stomach), and coadministration of H_2-receptor antagonists and proton pump inhibitors which increase stomach pH, is expected to decrease bioavailability of delavirdine. The currently recommended dose for delavirdine is 400 mg three times a day [7].

Since many drugs are metabolized by CYP 3A4, many drug–drug interactions are likely to occur. Some studies were already undertaken to investigate interactions between delavirdine and e.g., fluconazole, rifabutin and rifampin [8,9].

Delavirdine plasma concentrations seem, like protease inhibitors and nevirapine, to be of value in predicting efficacy and side-effects. In this light, it is likely that therapeutic drug monitoring of delavirdine will improve treatment with this drug.

Staton et al. described a method to quantify delavirdine in human plasma [10]. They reported the development of an assay that was linear in the range 10–9300 ng/ml. However, the therapeutic range is much larger (up to 17 000 ng/ml) [2,9,11,12].

We here report the development and validation of a high-performance liquid chromatographic assay with fluorescence detection for the quantitative determination of delavirdine in human plasma. The presented assay is useful for pharmacokinetic research and therapeutic drug monitoring in HIV-1 infected patients treated with delavirdine, as the whole therapeutic range of the drug is covered.

2. Experimental

2.1. Equipment

The high-performance liquid chromatography (HPLC) system consisted of a P100 solvent delivery pump (Thermo Separation Products, Fremont, CA, USA), an AS300 automatic sample injection device (Thermo Separation Products), an FP920 intelligent fluorescence detector (Jasco, Tokyo, Japan) and a Datajet integrator (Thermo Separation Products). The analytical column was a Zorbax SB C_{18} column (75×4.6 mm I.D., particle size 3.5 μm; Rockland Technologies, Newport, DE, USA) protected by a Chromguard C_{18} guard column (10×3 mm I.D.; Chrompack, Middelburg, The Netherlands). Analytical runs were processed by the Winner on Windows system software (version 2.0, Thermo Separation Products).

2.2. Chemicals

Delavirdine mesylate was kindly provided by Pharmacia & Upjohn (lot PNU90152T, Kalamazoo, MI, USA). Acetonitrile and methanol (both HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands). Citric acid monohydrate and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Distilled water was used throughout. Blank, drug-free plasma was obtained from the Central Laboratory of Blood Transfusion Service (Amsterdam, The Netherlands).

2.3. Preparation of standards

Stock solutions of delavirdine were prepared by dissolving the appropriate amount of the compound, accurately weighed, in methanol–acetonitrile (9:1, v/v) to yield a concentration of approximately 1 mg/ml. Drugs for interference analysis were obtained from the hospital pharmacy (Slotervaart Hospital, Amsterdam, The Netherlands), either as solutions for injection or after dissolving solid reference material in 50% (v/v) methanol (final concentration 500 μg/ml).

2.4. Sample pretreatment

For the preparation of calibration samples the stock solution of delavirdine was diluted with methanol. For the construction of each calibration curve 10 spiked plasma samples were analyzed in duplicate. Calibration concentrations of 50–50 000 ng/ml delavirdine in plasma were prepared in Eppendorf tubes by adding 50 μl diluted stock solution to 950 μl of human plasma. The solutions were mixed for 10 s. Subsequently, 200 μl of the plasma standards were mixed with 800 μl of acetonitrile for 30 s. The tubes were then centrifuged for 10 min at 10 500 *g*, and 800 μl of the clear supernatant was evaporated to dryness under a gentle stream of nitrogen at 40°C. The residues were then dissolved in 100 μl of mobile phase, mixed for 1 min and centrifuged for 10 min at 10 500 *g*. The clear supernatants were transferred to autosampler vials with inserts for HPLC analysis.

A second stock solution with separate weighing was used for the preparation of quality control samples (QCs) yielding concentrations of 103, 1027, 10 273 and 41 092 ng/ml of delavirdine in plasma. The QCs were processed identically to the calibration samples.

2.5. Chromatography

The chromatographic analysis was performed at ambient temperature on a C_{18} analytical column with a mobile phase composed of citrate buffer (25 m*M*)–acetonitrile (82:18, v/v). The pH of the water–acetonitrile mixture was adjusted to 2.7 using 2 *M* hydrochloric acid. Prior to use, air was removed by purging helium through the mobile phase. The excitation and emission wavelengths used were 300 and 425 nm, respectively. The flow-rate was maintained at 1.5 ml/min. Aliquots of 25 μl were injected.

2.6. Specificity and selectivity

The interference of endogenous compounds was investigated by the analysis of different blank plasma samples originating from six individuals. The following compounds, frequently used by HIV-infected patients, were investigated for interference with the analytical method (including sample pretreatment): amprenavir, co-trimoxazole, didanosine, efavirenz, fluconazole, folinic acid, ganciclovir, indinavir, lamivudine, methadone, nelfinavir, nevirapine, oxazepam, pyrazinamide, ranitidine, rifampin, ritonavir, saquinavir, stavudine, zalcitabine, zidovudine and zidovudine-glucuronide in a final concentration of 20 μg/ml in plasma.

2.7. Limit of detection and limit of quantitation

The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3.

The lower limit of quantitation (LLQ) was defined as the concentration for which the relative standard deviation and the percent deviation from the nominal concentration were less than 20%. The upper limit of quantitation (ULQ) was arbitrarily set at 50 000 ng/ml.

2.8. Accuracy, precision, linearity and recovery

Accuracy, between-day and within-day precisions of the method were determined by assaying six replicates of each of the QCs in three separate analytical runs. Accuracy was measured as the percent deviation from the nominal concentration. The within-day and between-day precision were obtained by analysis of variance (ANOVA) for each test concentration, using the analytical run as the grouping variable.

Linearity of three calibration curves was tested with the *F*-test for lack of fit, using a weighing factor of $1/\text{conc.}^2$ [13,14]. For the construction of each calibration curve 10 spiked plasma samples were analyzed in duplicate.

The average recovery of delavirdine over the concentration range of the standard curve was determined in three analytical runs by calculating the ratio of the slopes of a calibration curve in plasma and non-processed solutions.

2.9. Stability

Blank plasma samples were spiked with aliquots of diluted delavirdine stock solution to yield concentrations of 1027, 10 273 and 41 092 ng/ml. These samples were kept for 1 h at 60°C, 24 h at 25°C, 7 days at 4°C, 30 days at −30°C, and 30 days at −30°C including three freeze–thaw cycles. For each concentration and each storage condition six replicates were analyzed in one analytical run. The concentration of delavirdine after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

2.10. Analysis of patient samples

Plasma samples from an HIV-1-infected patient who ingested 400 mg of delavirdine after an overnight fast were assayed to establish steady-state pharmacokinetics of delavirdine. Twelve heparinized blood samples were drawn during 8 h. Furthermore, single plasma samples of 13 different patients were analysed with the currently reported methodology.

2.11. Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 6.1 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated P values were 0.05 or less.

3. Results and discussion

3.1. Chromatography and detection

The starting-point for the development of our method was the assay described by Staton et al. [10]. Reversed-phase chromatography was initially performed with a CN analytical column and a mobile phase consisting of phosphate buffer (25 mM, pH 6)–acetonitrile (2:1, v/v) [10]. We noticed, however, severe reconstitution problems with this mobile phase due to poor solubility of delavirdine at pH 6. Delavirdine is a weak base and the solubility largely depends on the pH of the solvent. From the literature we learned that peak plasma concentrations are up to 20 000 ng/ml or higher, so improving the solubility was necessary [2,4,11,12].

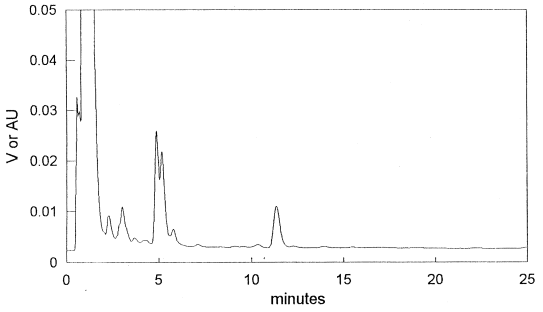

Fig. 2. Chromatogram typical for the analysis of delavirdine: blank plasma.

When using a pH below the pK_a value (approximately 3) solubility problems were solved. A Zorbax SB C_{18} column was then more appropriate. The phosphate buffer was replaced by a citrate buffer.

An acetonitrile content of 18% in combination with pH 2.7 of the mixture buffer–acetonitrile turned out to be optimal for separation of delavirdine from endogenous plasma compounds. Typical chromatograms of a blank plasma sample and of a spiked sample containing 4865 ng/ml delavirdine in plasma are shown in Figs. 2 and 3, respectively. The run time of the assay is 25 min. Maximal response was

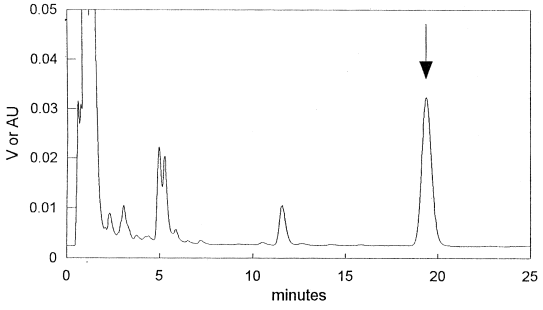

Fig. 3. Chromatogram typical for the analysis of delavirdine: a spiked plasma sample of 4865 ng/ml.

measured at an excitation wavelength of 300 nm and an emission wavelength of 425 nm.

No suitable internal standard was available. However, the assay gives satisfactory validation results without using an internal standard.

3.2. Sample pretreatment and recovery

Protein precipitation with acetonitrile and subsequent evaporation of the organic phase to concentrate the analyte, is a rapid, simple and effective sample pretreatment procedure. Recovery of delavirdine from spiked plasma samples by using this methodology is 93.8±2.3%.

3.3. Specificity and selectivity

Blank plasma from six different individuals showed no interfering endogenous substances eluting at the retention time (t_r) of delavirdine. Potentially co-administered drugs or metabolites tested had retention times that were different from delavirdine (t_r=19.2 min) (co-trimoxazole, t_r=30 min, pyrimethamine, t_r=28 min, oxazepam, t_r=31 min,) or were not detected with the described analytical method.

3.4. Limit of detection and limit of quantification

The LOD in plasma was 10 ng/ml. At this concentration the signal-to-noise ratio was 3. At 50 ng/ml the relative standard deviation and percent deviation from the nominal concentration were both less than 20%. Thus, 50 ng/ml was defined as the LLQ. At all other concentrations up to the ULQ (50 000 ng/ml) the relative standard deviation and percent deviation from the nominal concentration were always less than 15%.

In clinical practice the average steady-state peak and trough concentrations are approximately 16 and 7 μg/ml, respectively [11]. But, for example when delavirdine is combined with indinavir, steady-state peak concentrations are approximately 21 μg/ml [12]. Hence, we have extended the dynamic concentration range of this HPLC methodology by which it is now applicable in clinical pharmacokinetic studies and routine drug monitoring.

3.5. Validation: accuracy, precision, linearity and stability

Calibration curves proved to be linear in the range of 50–50 000 ng/ml with the use of the *F*-test for lack of fit (α=0.05) as an indicator of linearity of the regression model. For every calibration curve the calibration concentrations were calculated from the peak areas of delavirdine (see Table 1).

Accuracies and precisions for the quantitation of delavirdine in human plasma are listed in Table 2. Using the peak area in combination with a weighing factor ($1/\text{conc.}^2$) resulted in a minimal deviation from nominal concentrations.

The method proved to be accurate (average ac-

Table 1
Calibration curves: deviation from the nominal concentration and relative standard deviation for the analyte at all tested calibration concentrations[a]

Concentration of delavirdine (ng/ml)	Run 1 (n=2)		Run 2 (n=2)		Run 3 (n=2)		Mean 3 runs	
	Dev. (%)	CV (%)	Dev. (%)	CV (%)	Dev. (%)	CV (%)	n=6	CV (%)
50	+0.6	6.7	−0.2	4.8	+2.6	2.9	+1.0	3.7
100	+0.1	0.2	+2.7	8.1	−3.2	1.7	−0.1	4.7
250	−2.1	1.6	−2.8	0.3	−4.5	4.0	−3.1	2.3
500	−1.4	3.2	−5.1	0.1	−0.4	4.1	−2.3	3.3
1000	−1.7	0.2	−2.3	3.0	+0.1	–	−1.6	1.8
2500	+2.9	2.6	−0.1	0.7	−1.7	0.2	−0.5	1.5
5000	−5.4	0.6	−4.0	0.2	−5.9	0.6	−5.1	1.0
10 000	+1.9	0.7	+2.8	6.3	+0.2	4.6	+1.6	3.7
25 000	+4.0	1.4	+3.2	0.9	+8.1	1.2	+5.1	2.4
50 000	+3.7	0.6	+5.5	0.8	+4.6	1.5	+4.7	1.1

[a] CV=Coefficient of variation; Dev.=deviation from the nominal concentration; n=number of replicates.

Table 2
Accuracy and precision for the analysis of delavirdine in spiked human plasma samples

Concentration (ng/ml)	Accuracy (%)	Precision		n[a]
	Between-day	Within-day		
103	108.4	4.4	2.7	18
1027	110.5	2.9	0.2	18
10 273	108.3	3.4	0.6	18
41 092	103.7	3.7	3.7	17

[a] n=Total number of replicates in three analytical runs.

curacy at three concentrations 103.7–110.5% of the nominal concentrations) and precise (within-day precision ranged from 0.2–3.7% and between-day precision ranged from 2.9–4.4%). Correlation coefficients (r^2) of calibration curves were >0.998 as determined by least-squares analysis.

The stability of delavirdine under various conditions is shown in Table 3. One concentration (41 092 ng/ml) was below 90% (89.5%) after three freeze–thaw cycles were carried out, but the coefficient of variation in this case was also rather high (10.2%). All other conditions and concentrations tested showed that delavirdine is stable with concentrations of at least 90.4% of the initial concentration.

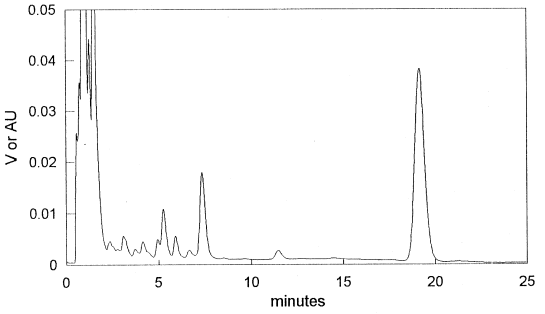

Fig. 4. Chromatogram typical for the analysis of delavirdine: a patient sample containing 7253 ng/ml.

3.6. Analysis of patient samples

The applicability of the assay for pharmacokinetic research in HIV-1-infected patients was demonstrated by measuring 13 patient samples (range: 528–18 882 ng/ml).

A typical chromatogram of a patient sample of 7253 ng/ml is shown in Fig. 4.

The plasma concentration–time profile of delavirdine in a patient after ingestion of 400 mg of delavirdine as determined by the currently described analytical method is shown in Fig. 5.

Table 3
Stability of delavirdine in spiked human plasma samples[a]

Storage conditions	Concentration (ng/ml)	Recovery (%)	CV (%)	n
1 h at 60°C	1027	101.3	2.0	6
	10 273	108.7	2.8	6
	41 092	96.3	5.0	6
24 h at 25°C	1027	100.3	7.3	6
	10 273	104.5	8.1	6
	41 092	99.3	3.2	6
7 days at 4°C	1027	98.4	1.3	6
	10 273	106.9	7.6	6
	41 092	101.7	3.0	6
30 days at −30°C	1027	103.1	4.3	6
	10 273	105.2	2.8	5
	41 092	90.4	10.7	6
Three freeze–thaw cycles	1027	102.8	2.4	6
	10 273	108.3	3.1	6
	41 092	89.5	10.2	6

[a] CV=Coefficient of variation; n=number of replicates.

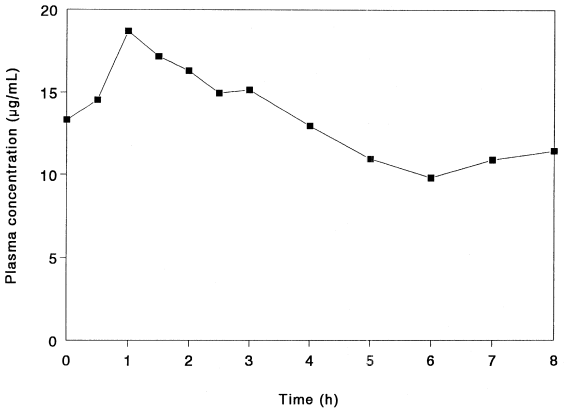

Fig. 5. Plasma concentration–time curve of delavirdine after oral administration of 400 mg of delavirdine to an HIV-1 infected patient (chronic use). The patient concomitantly used ritonavir (100 mg), indinavir (800 mg), lamivudine (150 mg) and zidovudine (300 mg), all twice daily.

4. Conclusion

In conclusion, a rapid, sensitive, specific and validated assay for the quantitative determination of delavirdine in human plasma is described. The applicability of the assay for pharmacokinetic research in HIV-1-infected patients is demonstrated by analysis of plasma samples from 13 HIV-1-infected patients at steady-state and a full pharmacokinetic profile during one dosing interval. The assay meets all current requirements for the validation of a bioanalytical method. This HPLC assay can be used for pharmacokinetic studies with delavirdine in HIV-infected patients and can readily be used to monitor delavirdine plasma concentrations in a hospital laboratory.

Acknowledgements

The authors would like to thank Pharmacia & Upjohn for supplying delavirdine reference compound. Furthermore, R. van Gijn and H. Rosing are kindly acknowledged for their assistance and suggestions.

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