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Determination of L-735 524, an human immunodeficiency virus protease inhibitor, in human plasma and urine via high-performance liquid chromatography with column switching

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Abstract

A method for the determination of an HIV protease inhibitor, L-735 524, in human plasma and urine is described. Isolation of the analyte and the internal standard from the matrices was achieved via multiple liquid–liquid extractions with methyl *tert.*-butyl ether. The analyte lacks significant UV absorption at wavelengths greater than 220 nm, hence a column switching system using a cyano and C_{18} column was used to further purify the extracts prior to UV detection at 210 nm. The assay has been found to be linear and has been validated over the concentration range of 5 to 500 ng/ml, when 1-ml aliquots of plasma or urine were extracted. The assay has been utilized to support human pharmacokinetic studies.

1. Introduction

Compound **I**, N-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[[2(*S*)-*tert.*-butylaminocarbonyl]-4-(3-pyridylmethyl)piperazino]-4(*S*)-hydroxy-2(*R*)-phenylmethyl-pentanamide (L-735 524, Fig. 1) is a potent and specific in vitro inhibitor of the human immunodeficiency virus Type 1 (HIV-1)-encoded protease [1–3]. HIV has been identified as the causative agent of acquired immune deficiency syndrome (AIDS) [4–7]. HIV-1 protease is utilized during the viral replication cycle to cleave a polyprotein into individual functional proteins, and thus transform the virus into an infectious form [8–10]. Inhibition of HIV-1 protease in vivo would be expected to prevent virus maturation, and therefore might be effective in the treatment of HIV-infected individuals [11,12]. An assay to determine the plasma and urine concentrations of **I** after oral dosing was needed to support human pharmacokinetic, safety and tolerability clinical studies. The development of a high-performance liquid chromatographic (HPLC) method using column switching [13,14] for the quantitation of **I** in plasma and urine is the subject of this paper.

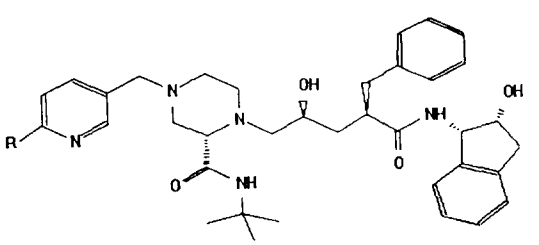

Fig. 1. Chemical structures of compound **I** (L-735 524) (R = H) and internal standard **II** (R = CH_3).

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2. Experimental

2.1. Materials

Compound **I** was obtained from the chemical data department of Merck Research Labs. (Rahway, NJ, USA). Compound **II** (Fig. 1), the internal standard, was prepared in the Medicinal Chemistry Department of Merck Research Labs. (West Point, PA, USA). Acetonitrile and methyl *tert.*-butyl ether (MTBE) (Omnisolve HPLC grade) were from EM Science (Gibbstown, NJ, USA). Drug-free human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA). Control urine was provided by volunteers in the Department of Drug Metabolism of Merck Research Labs.

2.2. Instrumentation

The HPLC system (Fig. 2) consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 410 pump (pump 1, P_1), a Perkin-Elmer Model 250 pump (pump 2, P_2), a Waters (Milford, MA, USA) WISP 715 automatic injector, a Valco (Houston, TX, USA) ten-port electrically actuated valve, and an Applied Biosystems (Foster City, CA, USA) 785 absorbance detector. The valve was controlled from the "timed events" output of pump 1. The analog output from the detector was connected to a PE-Nelson (Cupertino, CA, USA) Access-Chrom data system via a PE-Nelson 941 analog-to-digital interface.

2.3. Chromatographic conditions

The mobile phase for pump 1 consisted of a mixture of acetonitrile–water (34:66, v/v). The mobile phase for pump 2 was a mixture of acetonitrile–water (38:62, v/v). Each mobile phase was made 10 m*M* in orthophosphoric acid by the addition of 690 μl of 85% orthophosphoric acid per liter of mobile phase. The pH (apparent) of each of the mobile phases was adjusted to 7.5 with 10 *M* sodium hydroxide. The mobile phases were filtered through a nylon filter (0.20 μm) prior to use. Each mobile phase was delivered at a flow-rate of 1.2 ml/min.

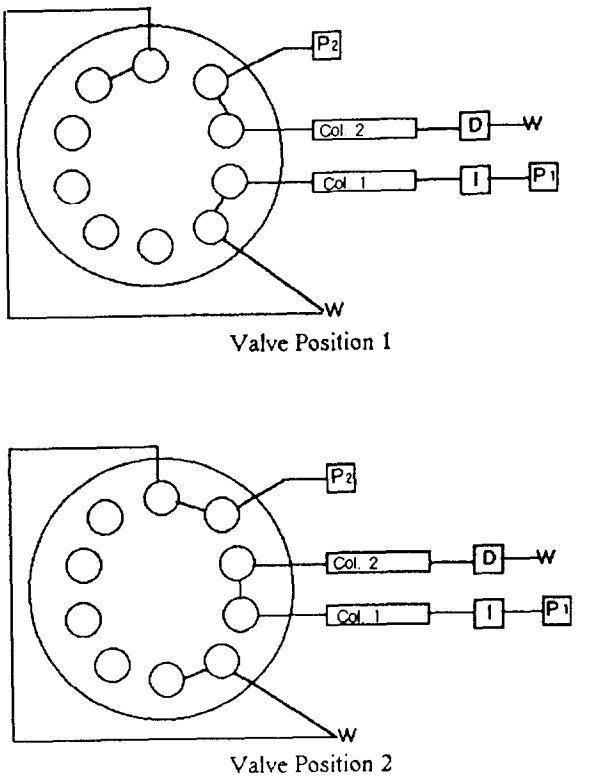

Fig. 2. Block diagram of HPLC column-switching system. I = Injector; P = pump; Col. = column; D = detector; W = waste.

Column 1 (Fig. 2) was a Zorbax SB-CN (5-μm particles with a pore diameter of 80 Å) cartridge column (80 × 4 mm) from Mac-Mod Analytical (Chadds Ford, PA, USA). Column 2 (Fig. 2) was an Inertsil ODS-2 (5-μm particles with a pore diameter of 150 Å) column (150 × 4.6 mm) packed by Keystone Scientific (State College, PA, USA). Column 1 was replaced after 150–200 injections, while the lifetime of column 2 was greater than 750 injections. The columns were used at ambient temperature (approximately 22°C).

The sample injection volume was 125 μl. Ultraviolet absorbance at 210 nm was used for detection.

2.4. Switching valve programming

The times at which the events on pump 1 were set to trigger the valve were determined daily using the following procedure. The valve was placed in position 2, as shown in Fig. 2. A piece of 0.007-in. I.D. tubing (1 in. = 2.54 cm) was connected in place of column 2.

A "window check" sample was prepared by pipetting 50 μl of a 10 μg/ml solution of **I** in acetonitrile and 25 μl of a 10 μg/ml solution of **II** in acetonitrile into a disposable glass culture tube, evaporating the solvent under a stream of nitrogen and reconstituting the residue in 200 μl of pump 1 mobile phase.

A 125-μl volume of the "window check" sample was then injected into the modified system. The time, t_1, at which the compound **I** peak began to elute (approximately 3.8 min), and the time, t_2, at which the compound **II** peak returned to baseline (approximately 5.2 min) were determined. The timed events were then set to place the valve in position 1 at the beginning of the run, switch the valve to position 2 at t_1, and return the valve to position 1 at t_2. Following this procedure, column 2 was reconnected, and the system was ready for use.

2.5. Preparation of standards

A 20 μg/ml stock solution of **I** was prepared by weighing 1.0 mg of reference material into a 50-ml volumetric flask, dissolving the compound in 25 ml of acetonitrile, and filling the flask to volume with water. A 2.0 μg/ml stock solution was prepared by diluting 5 ml of the 20 μg/ml solution to 50 ml with acetonitrile–water (50:50, v/v).

Working standards of 10, 8, 4 and 2 μg/ml were prepared by dilution of the 20 μg/ml stock with acetonitrile–water (50:50, v/v). Working standards of 1, 0.4, 0.2 and 0.1 μg/ml were prepared by dilution of the 2.0 μg/ml stock with acetonitrile–water (50:50, v/v). Working standard solutions were found to be stable for at least 1 month at room temperature.

Analysis standards were prepared by adding 50 μl of each working standard to 1 ml of drug-free plasma or urine. The resulting standards ranged in concentration from 5 to 500 ng/ml.

2.6. Sample extraction procedure

A 1-ml aliquot of plasma or urine (sample or standard) was pipetted into a 15-ml disposable glass centrifuge tube (Kimble, Vineland, NJ, USA). A 25-μl volume of a 10 μg/ml solution of internal standard (compound **II**) in acetonitrile was added and the contents of the tube were vortex mixed. A 1-ml volume of 0.1 *M* pH 9.5 borate buffer was added, the tube contents were vortexed, and 8.0 ml of MTBE were added. The tube was sealed with a PTFE-lined screw cap (Qorpak 5201; Fisher Scientific, Springfield, NJ, USA), tumble mixed on a Glas Col (Terre Haute, IN, USA) RD350 rotator for 5 min, and centrifuged at 1500 *g* for 5 min. The tube was then placed in a dry ice–acetone bath, the lower aqueous layer was frozen, and the entire upper organic layer was transferred to a clean centrifuge tube. A 1-ml volume of 10 m*M* HCl was added to the tube containing the organic layer. Following tumble mixing and centrifugation (as described above), the upper organic layer was aspirated to waste. The acid layer that remained was neutralized by the addition of 10 μl of 1 *M* sodium hydroxide, after which it was buffered to pH 9.5 by the addition of 1.0 ml of 0.1 *M* borate buffer. MTBE (8 ml) was added to the tube. After tumble mixing and centrifugation using the conditions described above, the lower aqueous layer was frozen in a dry ice–acetone bath, and the organic layer was transferred to a 100 × 16 mm glass culture tube. The tube was placed in a Turbo-Vap evaporator (Zymark, Hopkinton, MA, USA) set at 42°C and the solvent was evaporated under a stream of nitrogen. The resulting residue was reconstituted in 200 μl of pump 1 mobile phase and the sample was transferred to a polymethylpentene limited-volume insert contained in an autosampler vial. The vial was capped and placed in an autosampler tray

prior to injection into the column switching system.

3. Results

3.1. Assay specificity

Fig. 3 shows chromatograms of extracted control plasma, a plasma standard containing **I** (10 ng/ml) and **II** (250 ng/ml) and a plasma sample taken from a subject after receiving a 200-mg dose of **I**. Fig. 4 shows chromatograms of extracted control urine, a urine standard containing **I** (500 ng/ml) and **II** (250 ng/ml) and a urine sample collected between 0 and 3 h following the administration of 200 mg **I**. A comparison of Fig. 3A with Fig. 3B and Fig. 4A with Fig. 4B illustrates that no endogenous peaks co-elute with any of the analytes. The specificity of the method is further illustrated by the fact that all pre-dose plasma and urine samples from subjects

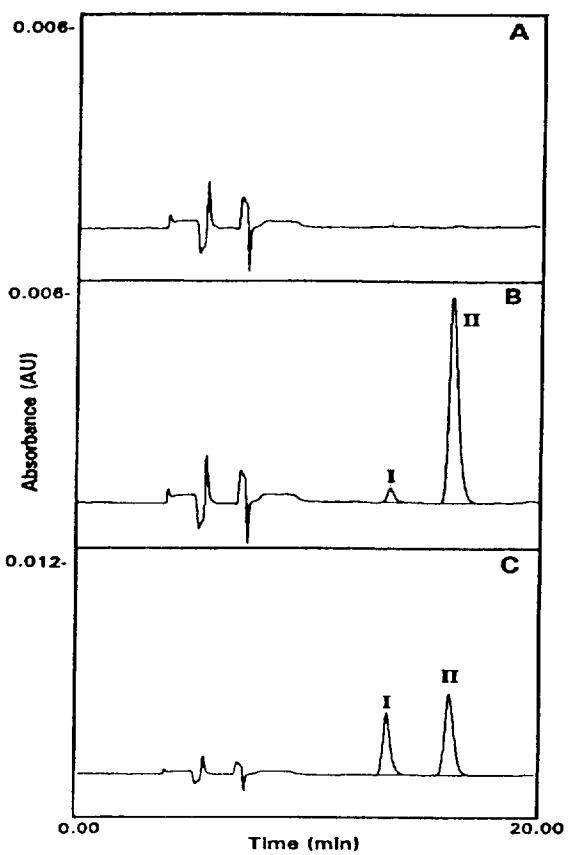

Fig. 3. Representative chromatograms of plasma. (A) Control human plasma, (B) plasma spiked with 10 ng/ml **I** and internal standard **II** (250 ng/ml), (C) plasma sample from human subject 1.5 h after administration of 200 mg **I**. Internal standard **II** added at 250 ng/ml. The concentration of **I** is equivalent to 120.8 ng/ml.

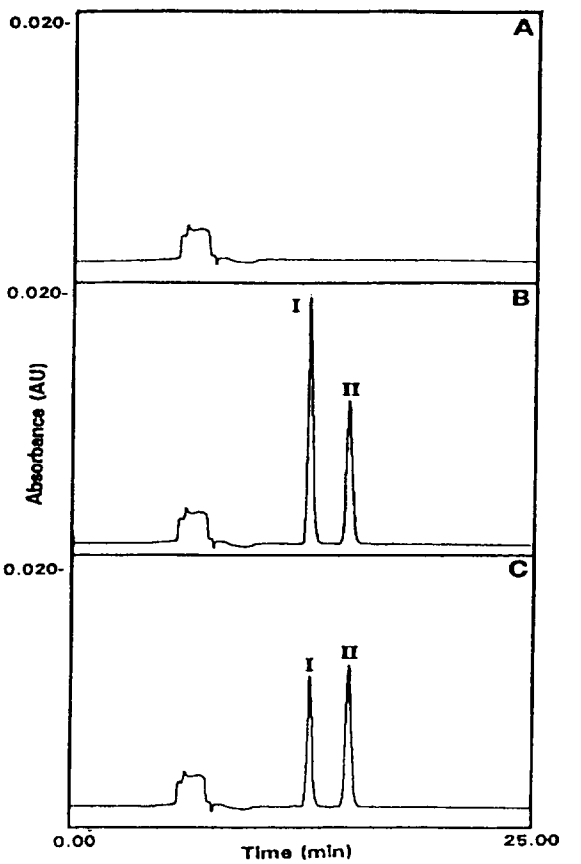

Fig. 4. Representative chromatograms of urine. (A) Control human urine, (B) urine spiked with 500 ng/ml **I** and internal standard **II** (250 ng/ml), (C) 0–3-h urine collection from a human subject obtained after the administration of 200 mg **I**. Sample diluted 1:200 prior to the addition of internal standard **II** at a concentration of 250 ng/ml. The concentration of **I** is equivalent to 52.05 μg/ml.

Table 1
Extraction recovery of **I** from human plasma and urine

Concentration (ng/ml)	Mean ($n = 5$) recovery (%)[a]	
	Plasma	Urine
5.0	84.7 (2.2)	68.1 (7.3)
10.0	81.2 (2.9)	70.0 (3.6)
20.0	79.5 (1.0)	69.0 (7.1)
50.0	82.0 (0.7)	66.0 (8.3)
100.0	78.3 (2.9)	66.0 (4.9)
200.0	84.3 (6.5)	69.0 (5.6)
400.0	81.4 (3.2)	71.0 (5.0)
500.0	78.3 (5.0)	67.0 (13.3)

[a] Values in parentheses are R.S.D.s.

involved in clinical trials were free of interfering peaks.

3.2. Linearity

Weighted (weighting factor = $1/y$ where y = peak height ratio) least-squares regression calibration curves, constructed by plotting the peak height ratio of **I**/**II** vs. standard concentration yielded coefficients of regression typically greater than 0.999 over the concentration range of 5 to 500 ng/ml of **I**. The use of the weighted least-squares regression resulted in less than a 10% deviation between the nominal standard concentrations and the experimentally determined standard concentrations calculated from the regression equation.

3.3. Extraction recovery

The recovery of the extraction procedure was determined by comparing the responses of the working standards of **I** injected directly into the HPLC system with those of extracted plasma and urine standards. The results (Table 1) indicate that the mean recovery of the extraction procedure over the concentration range of 5–500 ng/ml **I** is 81.2% for plasma and 68.3% for urine.

3.4. Assay precision and accuracy

Replicate standards ($n = 5$) were analyzed to assess the within-day variability of the assay. The mean assayed concentrations as well as the mean accuracy and relative standard deviations (R.S.D.s) of the analyses are shown in Table 2.

Quality control samples containing concentrations of 15 and 350 ng/ml **I** in plasma and urine

Table 2
Intraday precision and accuracy of the assay as assessed by the replicate ($n = 5$) analysis of standards

Nominal standard concentration (ng/ml)	Plasma			Urine		
	Mean ($n = 5$) analyzed standard concentration (ng/ml)	Accuracy (%)[a]	R.S.D. (%)	Mean ($n = 5$) analyzed standard concentration (ng/ml)	Accuracy (%)[a]	R.S.D. (%)
5.0	5.4	108.0	3.7	5.5	110.0	4.4
10.0	10.5	105.0	6.4	10.6	106.0	3.5
20.0	20.6	103.0	3.3	19.7	98.5	4.4
50.0	48.6	97.2	2.1	47.7	95.4	3.7
100.0	98.9	98.9	4.8	98.6	98.6	5.3
200.0	202.4	101.2	3.1	197.8	98.9	2.3
400.0	399.8	100.0	1.1	407.4	101.9	1.6
500.0	504.7	100.9	1.2	514.7	102.9	1.6

[a] Calculated as (mean analyzed concentration/nominal concentration) · 100.

Table 3
Inter-day variability of the assay as assessed by R.S.D.s of low- and high-concentration quality control samples

Nominal concentration (ng/ml)	Plasma[a]		Urine[b]	
	Mean (n = 46) analyzed concentration (ng/ml)	R.S.D. (%)	Mean (n = 17) analyzed concentration (ng/ml)	R.S.D. (%)
15.0	14.8	5.8	15.2	2.7
350.0	342.5	3.2	336.3	2.4

[a] Over a period of 42 days.
[b] Over a period of 17 days.

were prepared and frozen (−20°C) in 1-ml aliquots. These quality controls were analyzed over periods of 42 days (plasma) and 19 days (urine) to assess the inter-day variability of the assay. The results (Table 3) indicate that the inter-day variability of the assay, as measured by the R.S.D. is less than 6%. The results also indicate that frozen plasma samples containing **I** appear stable for at least 6 weeks, while urine samples are stable for at least 2 weeks.

3.5. Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded an within-day R.S.D. of less than 10% and an within-day accuracy between 90 and 110% of nominal concentration, was 5 ng/ml in plasma or urine.

4. Discussion

In vitro experiments have demonstrated that a concentration of less than 100 nM (61.4 ng/ml) **I** was required to inhibit the spread of HIV-1 in cell culture by at least 95%. Hence, an assay with low ng/ml sensitivity was needed to support human pharmacokinetic studies.

The ultraviolet–visible spectrum of **I** obtained in a solution of acetonitrile–10 mM phosphate buffer (pH 7) (50:50, v/v) exhibited maxima at 262 nm and <200 nm. The molar extinction coefficient of the 262-nm band was 3060 M^{-1} cm^{-1}. The spectrum was found to be unaffected by pH changes. The compound was not found to exhibit fluorescence.

The relatively low molar absorptivity at 262 nm indicated that an assay with low nanogram sensitivity using this wavelength for detection was not likely. Since the UV spectrum of **I** revealed that the molar extinction coefficient of the molecule at 210 nm was approximately 29 000 M^{-1} cm^{-1}, it was decided to develop an assay with UV detection at this low wavelength. In order to develop an assay at 210 nm, a relatively non-specific wavelength for detection, an extensive sample clean-up was required prior to injection into the HPLC system.

Development of a suitable extraction scheme began with a study of the extractability of **I** from buffers over a pH range of between 1 and 12. The pyridine ring in the molecule has a pK_a estimated to be between 5 and 6. It was found that **I** was quantitatively extracted with MTBE from solutions with a pH greater than 7, where the pyridine moiety would be un-ionized, and no recovery was observed from solutions whose pH was less than 3, where the pyridine ring would be expected to be fully ionized. Based on this finding, plasma and urine samples spiked with **I** and buffered at pH values between 7 and 12 were extracted and chromatographed under reversed-phase conditions on a C_{18} column. The resulting chromatograms contained endogenous peaks that co-eluted with the analyte of interest. These peaks could not be separated from the peak of interest through mobile phase or column

manipulations. The addition of a back extraction step into acid, with a subsequent re-extraction into MTBE significantly reduced the interference from plasma and urine components, but low-level, co-eluting endogenous peaks were still present in the extracts of samples when detection at 210 nm was used.

An observation was made that the pattern of the endogenous peaks appeared different on a cyano column operated in the reversed-phase mode, as compared to that on a C_{18} column. The peaks which interfered with the detection of **I** on the cyano column apparently were different than those that interfered on the C_{18} column.

In order to take advantage of the different selectivity of the cyano and C_{18} columns, a column switching system [13,14] was employed. The system (Fig. 2), configured around a ten-port valve, was designed to operate as follows. The system was initially set such that the output of the cyano column (column 1) was directed toward waste (valve position 1). The extracted sample was injected into the system. As **I** began to elute from the cyano column, the valve was switched to position 2. Compounds **I** and **II**, which was only partially resolved from **I** on the cyano column, were transferred by means of the switching valve to the C_{18} column (column 2). After **II** eluted from the cyano column, the valve was returned to position 1. Compounds **I** and **II** were resolved from each other and endogenous interferences on the C_{18} column while other endogenous components from the cyano column were directed toward waste. By combining the different selectivities provided by the cyano and C_{18} columns, **I** and **II** could be detected under interference free conditions at 210 nm. Additionally, band broadening was minimized in the present configuration, as the mobile phase used with the cyano column was weaker than that used with the C_{18} column. The lack of band broadening contributed to the fact that a limit of quantification of 5 ng/ml could be achieved with the column-switching system. Similar methods employing column switching to enable short-wavelength UV detection of analytes extracted from biological matrices have been previously reported [15,16].

Although the recovery of the extraction procedure was slightly less for urine as compared to plasma, it was constant over the range of the standard curve. Additionally, the precision and accuracy of the urine assay were found to be comparable to those of the plasma assay, indicating that the lower recovery did not adversely affect the overall performance of the assay with urine as the matrix.

The described assay has been used to analyze clinical samples obtained after oral administration of **I**. The plasma levels of **I** that resulted after the administration of 200 mg of **I** to three volunteers are presented in Table 4. The corresponding urinary recoveries of unchanged **I** are shown in Table 5.

5. Conclusions

An HPLC assay using column switching has been developed for the determination of **I** in human plasma and urine. The method has been found to be precise, accurate and suitable for the

Table 4
Plasma concentrations (ng/ml) of **I** following single-dose administration of 200 mg to selected healthy volunteers

Subject	Time (h)					
	0	0.5	1.0	2.0	4.0	8.0
1	nd	1251.0	870.9	205.2	70.7	10.7
2	nd	1142.3	1340.6	433.0	152.0	22.1
3	nd	785.4	622.0	193.5	67.7	8.3

nd = Not detected.

Table 5
Urinary recovery of **I** following single-dose administration of 200 mg to selected healthy volunteers

Subject	Urinary recovery (mg) over time interval					Total recovery	
	0–3 h	3–6 h	6–12 h	12–24 h	24–48 h	mg	% Dose
1	12.49	1.03	0.42	0.16	0.02	14.12	7.02
2	12.26	0.37	0.59	0.16	0.04	13.42	6.67
3	12.05	0.79	0.75	0.14	0.04	13.77	6.84

analysis of plasma and urine samples. The assay has been successfully applied for the analysis of over 3000 clinical samples from human pharmacokinetic studies.

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