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High-performance liquid chromatographic determination of a potent and selective HIV protease inhibitor (L-735,524) in rat, dog and monkey plasma

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Abstract

A high-performance liquid chromatographic method coupled with liquid–liquid sample extraction and ultraviolet detection has been developed for the quantification of L-735,524 (I), a potent, highly selective and orally bioavailable inhibitor of recombinant human immunodeficiency virus (HIV) protease in rat, dog and monkey plasma. The present method is reproducible and reliable with limits of quantification of 25, 12.5 and 6.25 ng/ml, respectively for rat, dog and monkey plasma. The standard curve was linear over the range of 6.25–2000 ng/ml in the biological fluid. The mean coefficients of variation for concentration within the range of standard curve were 7.94, 6.91 and 4.52%, respectively, for intra-day analysis and 5.58, 9.27 and 5.45%, respectively, for inter-day analysis. The recoveries of I and L-707,943 (II), an analog of I used as the internal standard, from plasma samples were all over 88% through the extraction procedure. I and II are stable in mobile phase over a 48-h period while waiting for injection at ambient temperature and over a 144-h period in rat, dog and monkey plasma while stored at −20°C. Aqueous solubility of I is pH dependent, 60 mg/ml at pH 3.5 and 0.3 mg/ml at pH 4.8. The analytic procedures described in this report have been successfully employed to quantify the concentration of I in rat, dog and monkey plasma and provide the kinetic information for toxicological and pharmacological studies.

1. Introduction

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), encodes at least three enzymes: protease, reverse transcriptase and endonuclease [1]. The HIV protease is essential to cleave the polyprotein during virus maturation. Several observations suggest that the enzyme would be a good target for antiviral therapy [2,3]. L-735,524 (Fig. 1; I; N-(2-(*R*)-hydroxyl-1-(*S*)-indanyl)-2(*R*)-phenyl-methyl-4(*S*)-hydroxyl-5-(1-(4-(3-pyridyl methyl)-2(*S*)-N′-(*tert*-butyl carboxamido)piperazinyl))-pentane amide), a potent (IC_{50} = 0.4 n*M*), highly selective and orally bioavailable inhibitor of HIV protease in vitro [4], is currently under investigation for clinical use in the treatment of human acquired immunodeficiency syndrome (AIDS). A specific and sensitive analytical method is necessary to study the pharmacokinetics of I in laboratory animals to provide the kinetic information for toxicological and pharmacological studies. The present method involves liquid–liquid sample extraction

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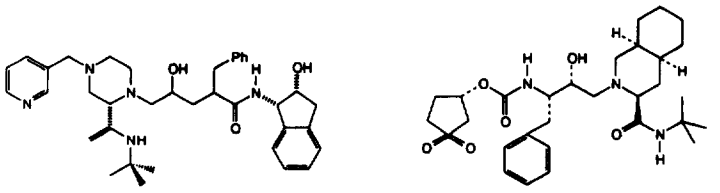

L-735,524 (I) L-707,943 (II, I.S.)

Fig. 1. Chemical structures of L-735,524 (I) and L-707,943 (II).

and reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet detection. Application of the developed method was demonstrated by quantifying I in rat, dog and monkey plasma following intravenous or oral administration of I.

2. Materials and methods

2.1. Chemicals

I and II were synthesized at Merck Research Laboratories (West Point, PA, USA). Acetonitrile, phosphoric acid (85%) and triethylamine (TEA) were obtained from Fisher Scientific Company (Fair Lawn, MI, USA). Diethyl ether was purchased from Burdick & Jackson Baxter (Muskegon, MI, USA).

2.2. Standard solution

Working standards for I were prepared by diluting a stock solution of 1.0 mg/ml in acetonitrile to 200, 20, 10 and 2.5 μg/ml. Compound II at a concentration of 150 μg/ml in acetonitrile served as the internal standard stock solution.

2.3. Apparatus

The high-performance liquid chromatographic system consisted of three components; a WISP Model 710B automatic sample injector with 96-sample card reader (Waters Assoc., Milford, MA, USA), 8700XR gradient pump with SP 8500 dynamic mixer (Spectra Physics, San Jose, CA, USA) and ABI analytical Kratos Spectroflow Model 785A programmable absorbance ultraviolet detector (Applied Biosystem, Foster City, CA, USA) operating at 220 mm. A 250 nm × 4.6 mm I.D. Zorbax RX-C_8 packed column coupled with inline guard column of 5 μm, 15 mm × 3.2 mm I.D. RP-8 (Brownlee Labs., Santa Clara, CA, USA). The mobile phase consisted of acetonitrile–0.015 *M* phosphoric acid (24:76, v/v), adjusted to pH 3.2 with triethylamine (TEA). The flow-rate was 1.5 ml/min.

2.4. Sample preparation

Plasma samples were prepared by mixing 200, 1000 or 500 μl of rat, dog, or monkey plasma with 10 μl of internal standard II solution (150 μg/ml) in a 100 mm × 12 mm screw-cap (PTFE liner, Kimble, Vineland, NJ, USA) test tube. After vortex-mixing, the sample was extracted with 5.0 ml of diethyl ether by shaking for 10 min on an Eberbach shaker. After centrifugation for 5 min, at 4500 *g*, 4.5 ml of the organic phase was transferred to a 100 mm × 13 mm culture test tube and evaporated to dryness under nitrogen. The residue was reconstituted to 250 μl of acetonitrile–phosphoric acid buffer solution (20:80, v/v) and vortex-mixed for 1.0 min. An aliquot (200 μl) of the final solution was injected onto the HPLC system.

2.5. Preparation of standard curve

Calibration standards were prepared by adding 2.5–10 μl of an appropriate working standard to 0.25, 1.0 or 0.5 ml of control rat, dog or monkey plasma. Nine calibration concentrations of I were used for the standard curve (6.25, 12.5, 25, 50,

100, 200, 500, 1000 and 2000 ng/ml) in each matrix. The samples were extracted and processed as described above. The concentration of I was calculated from the linear regression equation of the daily calibration curve constructed by plotting the ratio of peak height of I to that of the internal standard against the concentration of I in plasma.

2.6. Stability studies

The stability of I and II in mobile phase was performed by the analysis of I and II in mobile phase (1.0 μg/ml) at 0, 2, 4, 6, 8, 24 and 48 h by the HPLC method described above. The peak height of I and II from each time point were compared to that of 0 h which served as the 100% control. There were 18 spiked plasma samples taken from the control pool of animals including rat, dog and monkey, and the samples were stored at −20°C until analysis. A triplicate set of each plasma sample was thawed and analyzed every 24 h for 6 consecutive days. One set of the plasma samples was analyzed immediately after I and II were placed in plasma and were characterized by comparing the peak height of I and II from each set of plasma samples to the control plasma samples.

2.7. Biological samples

Two groups of adult male Sprague–Dawley rats, weighing from 250 to 400 g (Charles River, Wilmington, MA, USA) with free access to food and water, were used in this study. Under light pentobarbital anesthesia (40 mg/kg intraperitoneally), a cannula for blood sampling was implanted in the right jugular vein one day prior to the experiment. The animals received I orally or intravenously at 10 or 2 mg/kg, and blood samples were drawn via the cannula at specific time intervals over a 6-h period.

Three male beagle dogs (8–10 kg) and three Rhesus monkeys (3–5 kg) with free access to food and water were also used in this study. The animals received the compound orally or intravenously at 10 or 2 mg/kg in a cross-over fashion and blood samples were drawn via jugular or femoral vein at a specific time intervals over a 24- or 10-h period. All plasma samples were stored at −20°C and analyzed within 6 days.

3. Results and discussion

An HPLC method with ultraviolet detection combined with diethyl ether liquid–liquid samples extraction has been developed for the quantification of I in rat, dog and monkey plasma. The effects of pH and molarity of the mobile phase on the retention time were investigated in order to optimize chromatographic conditions suitable for sample analysis. The optimization processes were carried out by alternately varying the pH (2–4.5) and the molarity (0.015–0.12 *M*) of phosphoric acid buffer. The aqueous solubility of I is pH dependent, 60 mg/ml at pH 3.5 and 0.3 mg/ml at pH 4.8 (Fig. 2). Therefore, as expected, the effect of pH values on the retention time of I was found to be significant. For example, increasing the pH of the mobile phase from 2.0 to 4.5, caused a 10-fold increase in the retention time of I, while there was only a slightly effect on the retention time of II (Fig. 3). This can be explained by the chemical structure of I, it contains three potential sites of protonation. One located

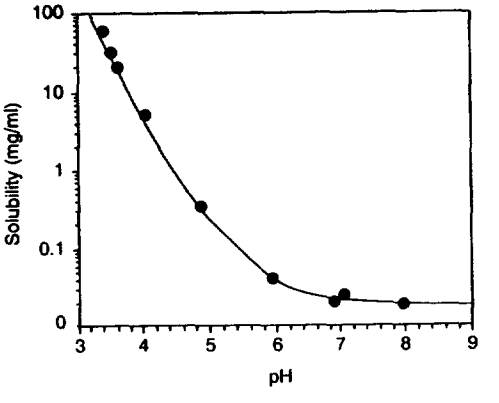

Fig. 2. Effect of pH on the aqueous solubility of I.

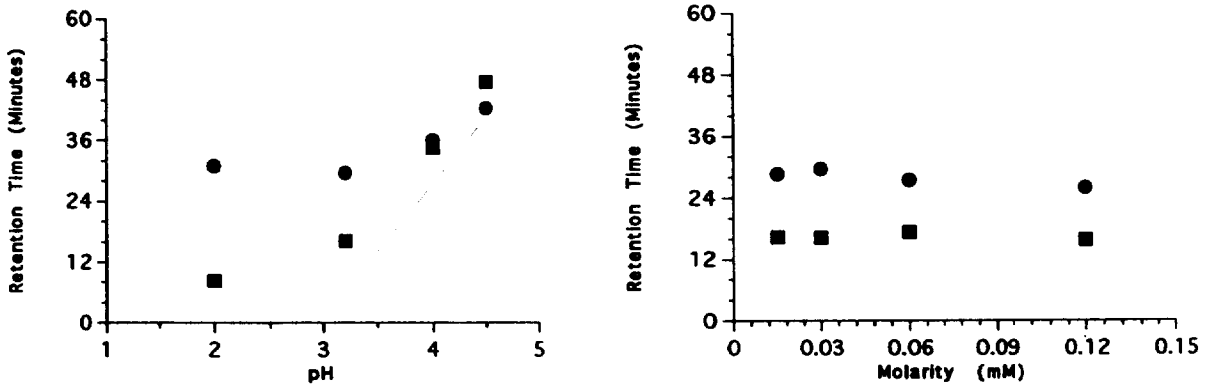

Fig. 3. Effects of pH and molarity on the retention time of I (■) and II (●).

on the pyridyl ring and the other two located on the piperazine ring. These basic nitrogens characterize the chemical ionization, when I is placed in an acidic environment such as pH = 2.0 mobile phase, two of the three nitrogens are fully protonated and the polarity of the compound will be increased. Therefore, the retention of I in the HPLC column will be shortened. When the pH value of the mobile phase is increased to 4.0, only one basic nitrogen ring located on the 4th position of the piperazine ring is full protonated. The polarity of I is decreased, increasing the retention time and making I inseparable from II. It is also interesting to note that the elution order of I and II is reversed when the pH value of the mobile phase is changed from pH 4.0 to 4.5. The effect of the molarity of phosphoric acid buffer was negligible.

Under the chromatographic conditions described in the experimental section, I and II were completely separated with respective retention times of 12.0 and 22.0 min. The limit of quantitation was determined by division of the lowest concentration of the standard curve by the plasma volume used in the HPLC assay. These values were 25, 6.25 and 12.5 ng/ml, respectively for rat, dog and monkey plasma. Fig. 4 shows representative chromatograms of control (A) and dosed (B) rat, dog and monkey plasma. No interfering peaks at the retention times of I and

Table 1
Recovery of I from rat, dog and monkey plasma following a liquid–liquid sample extraction by diethyl ether

Concentration (μg/ml)	Recovery (%)		
	Rat	Dog	Monkey
0.0125	90.2 ± 2.48	88.4 ± 0.75	94.9 ± 2.94
0.1	94.3 ± 4.13	90.8 ± 4.09	91.4 ± 3.04
1.0	92.6 ± 4.06	92.2 ± 1.96	93.1 ± 2.85

Table 2
Validation data for the determination of I in rat, dog and monkey plasma

Species	Linearity			Precision (%)	
	Slope	Y_{int}	r^2	Intra-day C.V.	Inter-day C.V.
Rat	3.58 ± 0.84	0.023 ± 0.0024	0.998 ± 0.001	7.94 ± 6.68	5.58 ± 2.04
Dog	3.57 ± 0.69	0.026 ± 0.0018	0.998 ± 0.001	6.90 ± 2.87	9.27 ± 5.59
Monkey	2.07 ± 0.43	0.026 ± 0.009	0.9997 ± 0.002	4.52 ± 3.18	5.45 ± 4.98

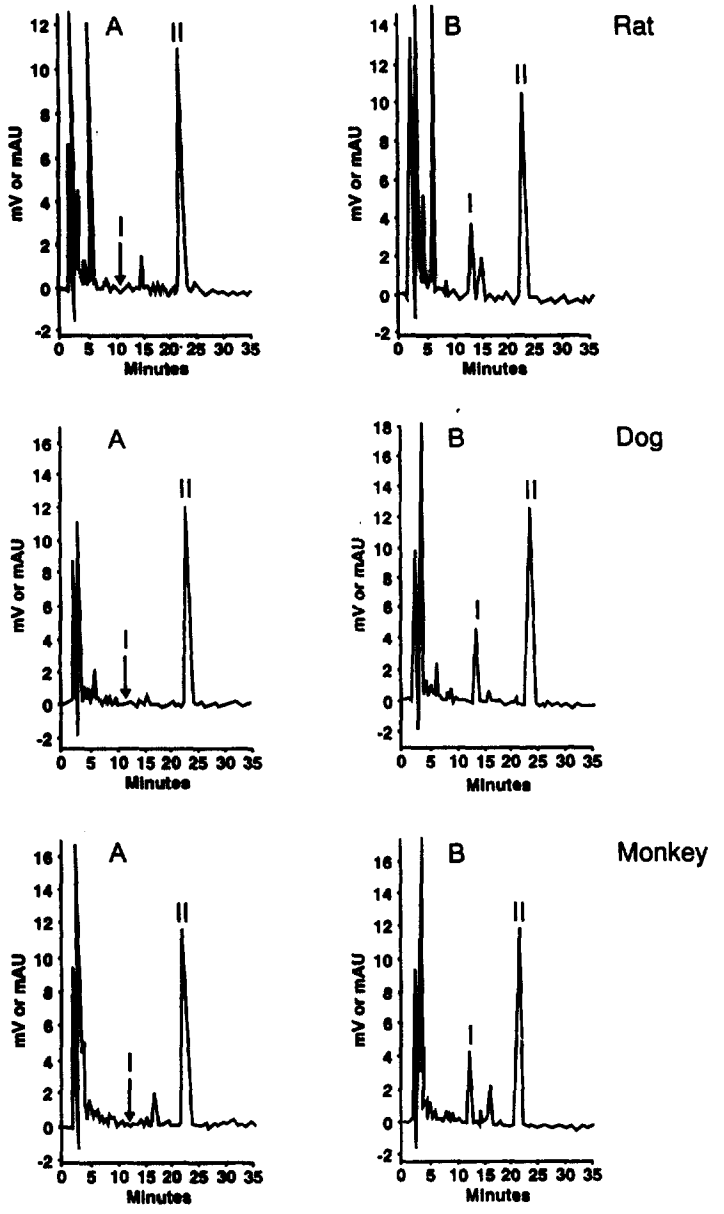

Fig. 4. Typical chromatograms of I and II in rat, dog and monkey plasma. (A) Sample containing 150 ng/ml of II rather after intravenous administration of 2 mg/kg I, (B) control plasma.

II were seen in control samples of any matrix. The diethyl ether liquid–liquid extraction has been found to be superior, since it yielded clean chromatograms (Fig. 2) and good recoveries of I and II. The recoveries of I and II in control rat, dog and monkey plasma at concentrations ranging from 12.5–1000 ng/ml were all over 88% (Table 1).

Standard curves ranged from 6.25 to 2000 ng/ml were linear in rat, dog and monkey plasma, respectively. The mean standard curves of I (n = 6/concentration) in plasma were described in Table 2. The r^2 were 0.998, 0.999 and 0.9997, respectively for rat, dog and monkey.

Individually prepared replicate ($n = 4$) standards were analyzed to assess intra-day repro-

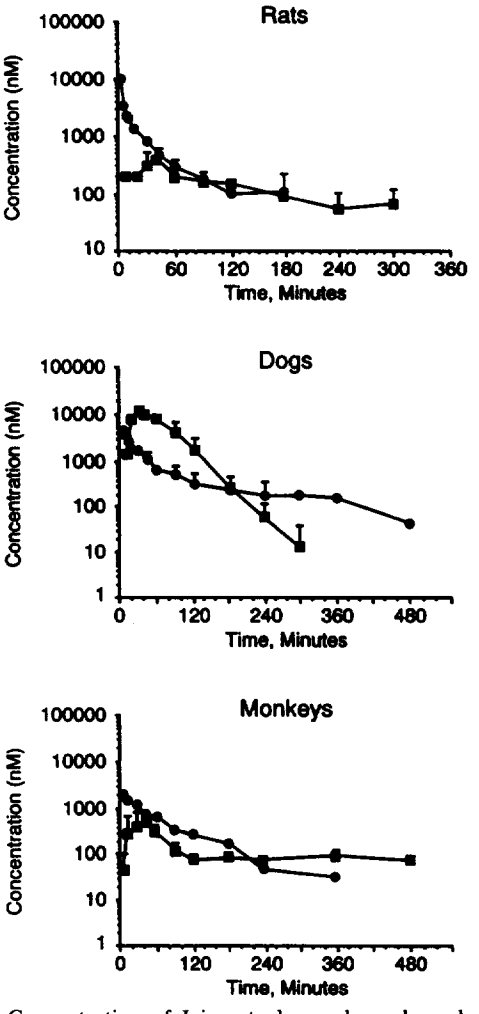

Fig. 5. Concentration of I in rat, dog and monkey plasma following intravenous (●) or oral (■) administration of 2 or 10 mg/kg I.

ducibility. All concentrations (50, 125, 200 and 1000 ng/ml) were included in the range of standard curve and tested. The mean C.V. values from the above test standard were 7.94, 6.90 and 4.52% for rat, dog and monkey, respectively. Inter-day reproducibility was assessed through the daily analysis of quality control (QC) standard. Three different concentrations (25, 200 and 2000 ng/ml) of QC standard solutions were prepared and assayed. The mean coefficients of variation for the QC standard of I in plasma were 5.58, 9.27 and 5.45, respectively for rat, dog and monkey (Table 2).

I and II were very stable in mobile phase. Almost 100% recovery was found over a 48-h period while waiting for injection at ambient temperature in an autosampler, and over a 144-h period in rat, dog and monkey plasma while stored at −20°C.

The method described has been successfully applied to the quantification of I in rat, dog and monkey plasma and provided significant contributions to the pharmacokinetics in laboratory animals for the Investigational New Drug (IND) application. Fig. 5 illustrates the mean concentrations of rats, dogs and monkeys following i.v. and p.o. administration. The absorption kinetics of I in rat, dog and monkey following an oral administration of 10 mg/kg of I are summarized in Table 3. Following an oral administration of I at 10 mg/kg, the bioavailability was estimated to be 23.8, 71.2 and 14.2% respectively, for rat, dog and monkey [5].

Table 3
Absorption kinetics of I in laboratory animals receiving an oral dose of 10 mg/kg

Species	C_{max} (μM)	T_{max} (min)	$[AUC]_0^\infty$ ($\mu M \cdot h$)	Bioavailability (%)
Rat ($n = 3$–4)	0.44 ± 0.14	35.0 ± 5.80	0.85 ± 0.20	23.9
Dog ($n = 3$)	11.4 ± 2.30	30.0 ± 0.00	12.5 ± 4.10	71.6 ± 2.80
Monkey ($n = 3$)	0.71 ± 0.24	65.0 ± 22.9	1.45 ± 0.29	14.2 ± 2.84

C_{max}: peak concentration; T_{max}: time reached peak concentration; $[AUC]_0^\infty$: areas under plasma concentration curve from 0 min to infinity.

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