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Stereospecific determination of an HIV aspartyl protease inhibitor, PNU-103017, in rat, dog and human plasma using a Pirkle-concept high-performance liquid chromatographic column

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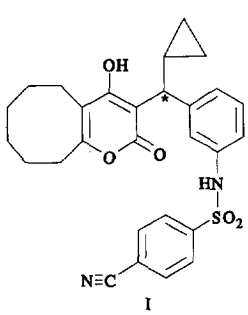
Abstract

A sensitive stereospecific high-performance liquid chromatographic assay for the quantitation of the enantiomers of 4-cyano-N-(3-(cyclopropyl-(5,6,7,8,9,10-hexahydro-4-hydroxy-2-oxo-2H-cycloocta(*b*)pyran-3-yl)methyl)phenyl)benzenesulfonamide (PNU-103017) (I), an HIV protease inhibitor, in plasma of rat, dog and human was developed. The procedure involved an acetonitrile-aided protein precipitation followed by solid-phase extraction (SPE) of I from plasma into ethanol. Stereospecific separation was accomplished on a Pirkle-concept chiral column (Regis S,S-Whelk-01, 250×4.6 mm I.D.) with a mobile phase of absolute ethanol–0.1% acetic acid in hexane (30:70, v/v). The eluate was monitored by UV absorbance (295 nm). Linear calibration curves were obtained in the range of 0.2 to 500 μ*M*, with a lower limit of quantitation of 0.1–0.2 μ*M* for both enantiomers in either rat, dog or human plasma. Intra- and inter-assay precision and assay accuracy were demonstrated to be acceptable for the stereoselective pharmacokinetic analysis of I in plasma.

Keywords: Enantiomer separation; PNU-103017; HIV aspartyl protease inhibitors

1. Introduction

4-Cyano-N- (3-(cyclopropyl- (5,6,7,8,9,10-hexahydro-4-hydroxy-2-oxo-2H-cycloocta(*b*)pyran-3-yl) - methyl)phenyl)benzenesulfonamide, PNU-103017 (I) (Fig. 1), is a selective HIV aspartyl protease inhibitor currently under clinical evaluation as a potential oral treatment of Acquired Immunodeficiency Diseases (AIDS) [1]. An achiral assay (HPLC-UV) using a Zorbax Rx-C8, 150×4.6 mm I.D., 5 μm (MacMod Analytical, Chadds Ford, PA, USA) with a mobile



I

Fig. 1. Chemical structure of PNU-103017 (I). Asterisk (*) denotes position of the chiral center. PNU-103264 (II) is the (*R*)-enantiomer and PNU-103265 (III) is the (*S*)-enantiomer.

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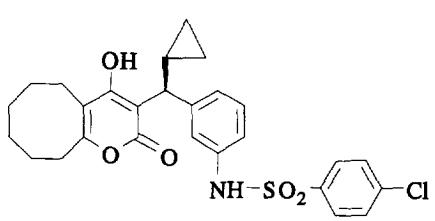
phase of 0.1% trifluoroacetic acid in water and acetonitrile (45:55, v/v) was developed to support the pharmacokinetic/toxicokinetic evaluation. Compound I is a racemate consisting of two enantiomers, PNU-103264, the (*R*)-enantiomer (II) and PNU-103265, the (*S*)-enantiomer (III). Since III was found to be more active in vitro, a need to define the pharmacokinetics of the individual enantiomers after single and multiple dosing of I has been raised for drug safety and efficacy considerations. Therefore, a chiral assay for the quantitation of the two enantiomers was developed. Described herein is the assay development and validation for the quantitation of the two individual enantiomers of I in the rat, dog and human plasma.

2. Experimental

2.1. Chemicals and reagents

Compounds II and III for the analytical standards used in the assay of the plasma samples and PNU-103262 (Fig. 2), the analytical internal standard (I.S.) (IV), were provided by Medicinal Chemistry of the Pharmacia and Upjohn (Kalamazoo, MI, USA). All the chemicals and organic solvents used were of HPLC or reagent grade. Purified water, Type I, was produced by a Milli-Q reagent water system (Millipore, Bedford, MA, USA). Pooled drug-free rat plasma was obtained from Sprague–Dawley rats within Pharmacia and Upjohn. Pooled drug-free beagle dog plasma was obtained from Buckshire (Perkasie, PA, USA). Pooled drug-free human plasma was obtained from Chemicon (Los Angeles, CA, USA). All plasma matrices used heparin as the anticoagulant.



IV

Fig. 2. Chemical structure of PNU-103262, the internal standard (I.S.) (IV).

2.2. Stock solutions

A stock solution of the I.S., IV, (3.89 m*M*) was prepared in absolute ethanol. The working I.S. solution (19.5 μ*M*) was prepared by dilution in acetonitrile. Both solutions were found to be stable for up to 4 months when stored in the dark at 1–5°C. The calibration standards were prepared from individual stock solutions in absolute ethanol containing 9.9 m*M* of II and III. A series of working standard solutions ranging in concentration from 0.99 μ*M* to 4.95 m*M* were prepared by serial dilution with absolute ethanol. These solutions were stable for up to 6 months when stored in tightly capped test tubes at 1–5°C.

Control standards were prepared in rat, dog and human plasma to mimic the conditions under which an unknown plasma sample would be stored and prepared, and to provide a means of evaluating the inter- and intra-assay accuracy and stability when stored at or below −15°C in plasma. A 3.96 m*M* stock solution of each of the enantiomers was prepared in absolute ethanol. The working control stock solutions were prepared by two serial dilutions of the stock solution to yield working control stock solutions of 0.4 m*M* and 0.04 m*M* of each enantiomer. These solutions were stored at 1–5°C.

The plasma control standard samples were prepared by aliquoting appropriate volumes of working stock solution into the appropriate volume of matrix. The plasma control standard concentrations for the rat and human were 396 μ*M*, 39.6 μ*M* and 3.96 μ*M*. The dog plasma control standard concentrations were 396 μ*M*, 19.8 μ*M* and 1.98 μ*M*. After thorough vortex mixing, aliquots of each control level were placed in appropriately labelled glass vials with polypropylene snap caps and kept at or below −15°C until time of assay when a vial of each concentration was thawed to room temperature and mixed gently but thoroughly before aliquoting.

2.3. Calibration standard preparation

The plasma calibration standards were prepared on the day of use by accurately pipetting a 50-μl aliquot of each working calibration standard solution into 100 μl of drug-free plasma from rat, dog or human in a 1.5-ml polypropylene conical centrifuge tube.

The plasma and working calibration standard solution were mixed well by vortex to give plasma samples with final concentrations of 0.0091, 0.198, 0.495, 0.991, 1.98, 4.95, 9.91, 19.8, 99.1, 198 and 495 μ*M* for each enantiomer. These samples constituted the calibration curve.

2.4. Preparation of samples

Samples were prepared for chromatography by a solid-phase extraction (SPE) method. Aliquots of 100 μl of control plasma standard or unknown plasma sample were placed in 1.5-ml polypropylene conical centrifuge tube and combined with 50 μl ethanol, 200 μl of 0.1 *M* KH_2PO_4 buffer solution and 100 μl I.S. working solution. Plasma calibration standards, which already had the ethanol component, were combined with 200 μl of 0.1 *M* KH_2PO_4 buffer solution, and 100 μl I.S. working solution. All samples were mixed briefly by vortex mixing. Centrifugation of the samples at 14 000 *g* for 2 min precipitated the proteins. The supernatant was loaded onto a Phenyl SPE column (50 mg/1.0 ml, Varian Sample Preparation Products, Harbor City, CA, USA) which had been pre-conditioned with 1 column volume (approximately 1 ml) of acetonitrile, followed by 1 column volume of 0.1 *M* KH_2PO_4 buffer solution, at a vacuum of approximately 86 kPa. When the sample had been loaded, the column was washed with 1 column volume of water followed by 0.2 ml of hexane–chloroform (2:1, v/v). The column was allowed to dry for at least 10 min under approximately 27 kPa vacuum. The enantiomers and I.S. were eluted into appropriately labelled 0.3 ml autosampler vials from the column with 150 μl of absolute ethanol followed by 200 μl hexane under slight vacuum. The vials were capped tightly and mixed briefly by vortex mixing. The autosampler vials were then placed in a cooled autosampler tray (1–4°C).

2.5. Chromatographic conditions

Chromatography of the enantiomers and I.S. was carried out on a normal-phase chiral column with UV detection. A Spectra-Physics 8810 pump (San Jose, CA, USA) was used to deliver a mobile phase of absolute ethanol–0.1% acetic acid in hexane (30:70, v/v) at a flow-rate of 1 ml/min to a S,S-Whelk-01 (25 cm×4.6 mm I.D.) column (Regis, Morton Grove, IL, USA). A Brownlee CN New-Guard (15×3.2 mm I.D., 7 μm) (Applied Biosystems, San Jose, CA, USA) pre-column was used. A 100-μl injection volume was used. Detection of the enantiomers and I.S. in the eluate was accomplished with a Spectra FOCUS UV detector set at 295 nm.

2.6. Calculations

Quantitation was accomplished by peak area ratio of drug to internal standard using an in-house chromatography software program on a Harris Night Hawk Computer System. The standard curve, along with a statistical evaluation of standards linear fit, was computed by the linear regression program available.

2.7. Method validation

Validation results for rat, dog or human plasma were derived from three analytical runs of calibration standards along with previously prepared three concentrations of control standard samples, assayed in triplicate, for each analytical run [2,3]. The inter-assay precision was obtained from the coefficients of variation (C.V.) of the control standards concentrations analyzed on three separate days and the intra-assay precision was determined from the C.V. of the control standards analyzed on the same day. The assay accuracy was evaluated by comparing the theoretical amounts with the determined amounts for the control standard samples. The low limit of quantitation (LLOQ) was the lowest concentration of the calibration standards at which the back-calculated value was ≤20% of the theoretical. Stability of the enantiomers in plasma at or below −15°C was determined by comparison of assays of plasma control standards maintained frozen for one, three and six months and the freshly made control standards. To further establish the stability of the enantiomers in plasma, plasma control standards that were freeze–thawed three times were assayed and the data compared to the freshly made control standards.

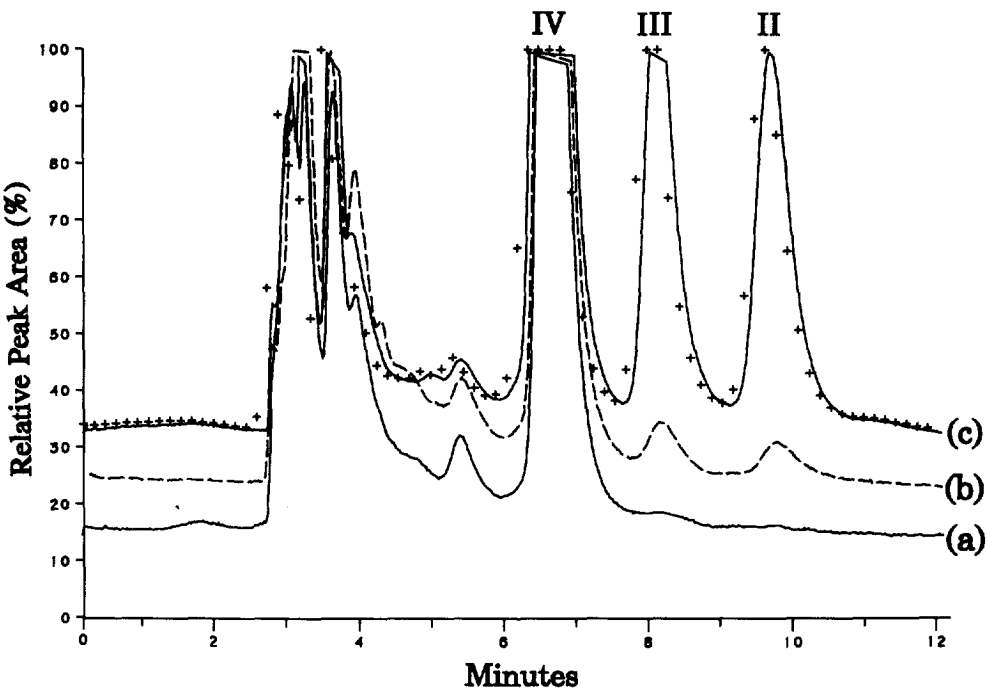


Fig. 3. Representative chromatograms of (a) a rat plasma blank with I.S., (b) a fortified plasma standard containing 0.5 μ*M* each of II and III, and (c) a post-dose plasma sample (day 1, 1 h) from rat No. 73 who received 240 mg/kg/day of I orally.

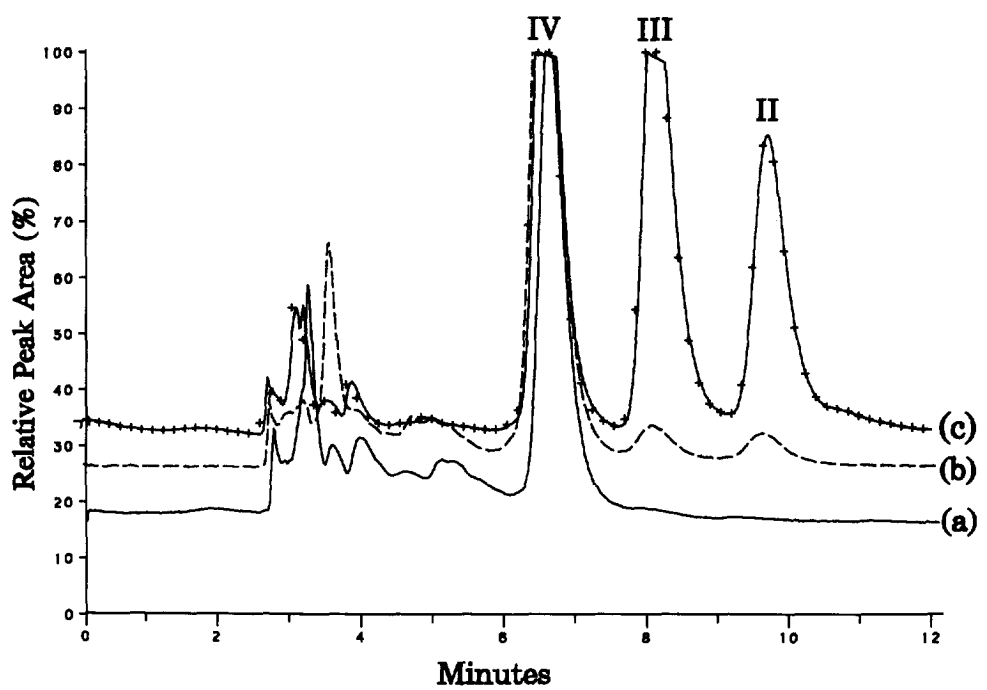


Fig. 4. Representative chromatograms of (a) a dog plasma blank with I.S., (b) a fortified plasma standard containing 0.5 μ*M* each of II and III, and (c) a post-dose plasma sample (day 1, 2 h) from dog No. 18 who received 100 mg/kg/day of I orally.

2.8. Application

The method was utilized in the evaluation of pharmacokinetics of the two individual enantiomers after single and multiple dose administration of I to the rat, dog and human for preclinical and clinical safety studies. Plasma samples from these studies were previously analyzed for concentrations of I using an achiral assay. Therefore, the accuracy of this chiral method could be evaluated by comparing the sum of the two enantiomer concentrations with the racemate concentrations.

3. Results

3.1. Chromatograms

The compounds of interest eluted within 12 min. Compound IV had a retention time of approximately 6.5 min. The enantiomers, III and II, eluted at approximately 8.2 min and 9.8 min, respectively. Figs. 3–5 show typical chromatograms of rat, dog and human plasma samples, respectively. Each overlaid chromatogram includes a matrix blank which shows that no endogenous substances in any of the matrices tested had the same retention time as the drugs or I.S.. Contamination of peaks II and III with metabolites are not considered likely since the sum of the concentrations of II and III were not consistently greater than the concentration of I obtained by an achiral method and were within experimental error (see Section 3.3).

3.2. Calibration curves and assay validation

The calibration standard data showed a linear relationship of y·I.S.$=ax$, force through the origin with no weighting, between the peak area ratio and concentration in the range of 0.1 to 500 μM for both enantiomers in all three matrices tested. The correlation coefficients (r) were ≥0.994 in rat, dog and human plasma for both enantiomers. The LLOQ for

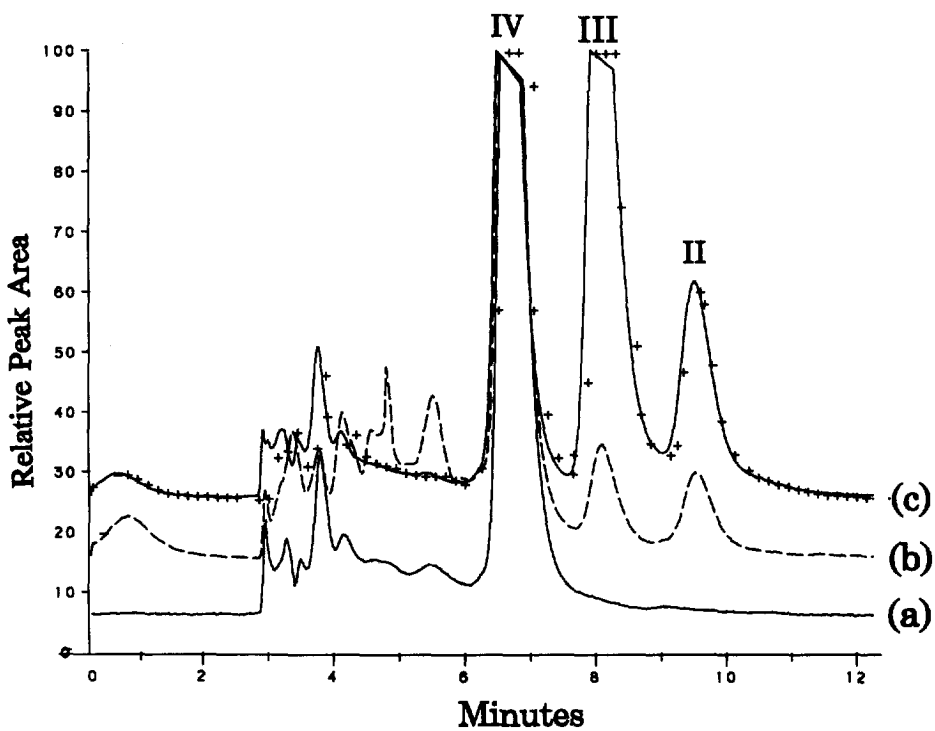


Fig. 5. Representative chromatograms of (a) a predose human plasma sample from subject No. 22, (b) a fortified human plasma standard containing 1 μM each of II and III, and (c) a post-dose plasma sample (day 1, 2 h) from subject No. 22 who received 400 mg of I orally.

both II and III ranged from 0.1 to 0.2 μ*M* in all three matrices tested.

As indicated in Tables 1 and 2, the intra-assay mean accuracy of control standards ranged from 87% to 108% with C.V. ranging from 0.5% to 13% and the inter-assay mean accuracy of control standards ranged from 93% to 107% with C.V. ranging from 2% to 10% for each enantiomer in three matrices evaluated. These values are within the acceptance criteria for analytical methods [2]. Stability of II and III in plasma kept at or below −15°C for up to six months and after three freeze–thaw cycles was found to be acceptable (data not shown). There was no evidence of conversion of II or III in plasma samples spiked with either II or III during sample preparation and chromatography as shown in chromatograms of extracted dog plasma spiked with either II or III (Fig. 6). Racemization of IV was not observed.

3.3. Applicability

The enantiomeric ratio (II/III) of I plasma concentrations in the rat, dog and human after single and multiple dose administration of the racemate de-

Table 1
Intra- and inter-assay precision and accuracy of II, the (*R*)-enantiomer, in rat, dog and human plasma

Comparison concentration (μ*M*)	Intra-assay ($n=3$)		Inter-assay ($n=9$)	
	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)
Rat plasma				
1.87	102	8	98	6
	96	4		
	97	3		
40.0	110	4	107	5
	108	4		
	97	3		
167	110	5	102	10
	103	5		
	94	13		
Dog plasma				
1.98	100	6	99	4
	98.6	1.6		
	97	4		
19.8	96.0	2.5	94.8	2.3
	94.4	2.3		
	94.2	2.6		
396	97.1	1.2	97.8	2.1
	97.9	2.7		
	98.5	2.6		
Human plasma				
3.96	101	1.7	95	6
	95	3		
	90	3		
39.6	104	10	101	6
	102	4		
	98	4		
396	101	0.5	93	8
	92	8		
	87.1	2.3		

Table 2
Intra- and inter-assay precision and accuracy of III, the (*S*)-enantiomer, in rat, dog and human plasma

Comparison concentration (μ*M*)	Intra-assay (*n*=3)		Inter-assay (*n*=9)	
	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)
Rat plasma				
1.87	99	9	95	7
	95	6		
	90	3		
40.0	98	5	97	5
	100	4		
	93	4		
167	101	5	102	6
	103	5		
	103	10		
Dog plasma				
1.98	97	5	96	4
	95	5		
	97.7	2.8		
19.8	96.3	2	95.1	2.8
	95.5	3		
	94	3		
396	99.0	1.5	99.2	2.3
	100.6	2.7		
	98.1	2.7		
Human plasma				
3.96	96.3	1.2	93	4
	91	4		
	91	4		
39.6	100	11	98	6
	99	4		
	96	4		
396	106	0.6	99	6
	98	5		
	94.2	2.3		

termined using this chiral assay are shown graphically in Fig. 7. Mean enantiomeric ratios of II/III plasma concentrations at each time point ranged from 1.22 to 3.06 in the dog, 0.44 to 0.80 in the rat, and 0.23 to 0.73 in the human, showing enantioselective pharmacokinetics of I after single and multiple dose administration of I. The details for these studies will be reported elsewhere [4]. The chiral assay results were also compared to I concentrations obtained from previous achiral assays. The bias between the sum of the two enantiomer concentrations and the concentration of the racemate was calculated to be within ±15% in 93% of the samples tested and within ±10% in 80% of the samples. This also demonstrated the accuracy of this chiral assay method.

4. Discussion

The S,S-Whelk-01 column was chosen after testing several chiral columns because of the excellent resolution of the enantiomers of I. The Whelk-01 chiral stationary phase is derived from 4-(3,5-dinitro benzamido)tetrahtdrophenanthrene, covalently bound to 5 μm 3-propyl silica, which allowed the separation of the two enantiomers of I without derivatization. The column has also proved to have an extremely good lifespan, beginning to show a deterioration in the chromatographic peak shapes after over 1200 injections. The initial mobile phase tested on the S,S-Whelk-01 column contained methanol. The peak shapes of the enantiomers and I.S. were broad, limiting the sensitivity of the assay. Changing the alcohol to ethanol improved peak shape and allowed quantitation into the sub-micromolar range.

The extraction recovery was poor when plasma samples were directly extracted using SPE. Since strong protein binding of I in plasma was found in all species (>99%) [5], a protein precipitation step with 100 μl acetonitrile prior to the SPE procedures was necessary to improve the extraction recovery. The supernatant was then purified through the SPE column, yielding a overall extraction recovery of 85% to 95% and a clean chromatogram with no interfering peaks at the retention times of II, III and IV. The 50 mg/ml Phenyl SPE column allowed the use of a small volume of elution solvent (150 μl of ethanol and 200 μl of hexane), which was concentrated enough to be directly injected into the HPLC system without evaporation and reconstitution procedures [6]. Drying the SPE column before elution was also an important step as the eluate containing even a small amount of water would deteriorate the peak shape of II, III and IV.

In conclusion, the assay presented was simple and shown to be sufficiently sensitive, precise and accurate for the quantitation of the enantiomers of I in rat,

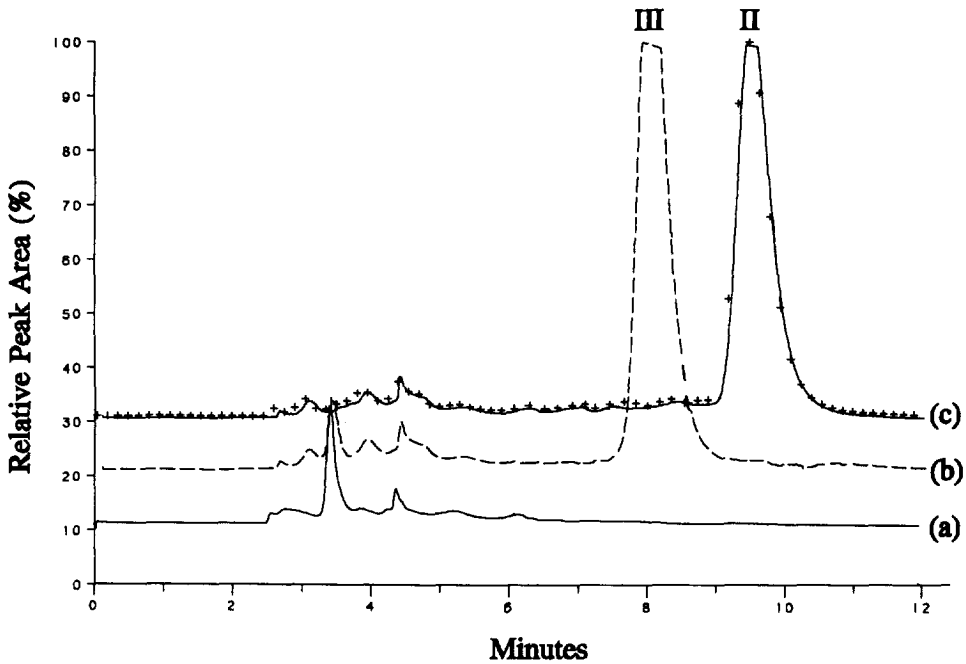


Fig. 6. Chromatograms of (a) extracted drug-free dog plasma, (b) extracted dog plasma fortified with 100 μ*M* III and (c) extracted dog plasma fortified with 100 μ*M* II.

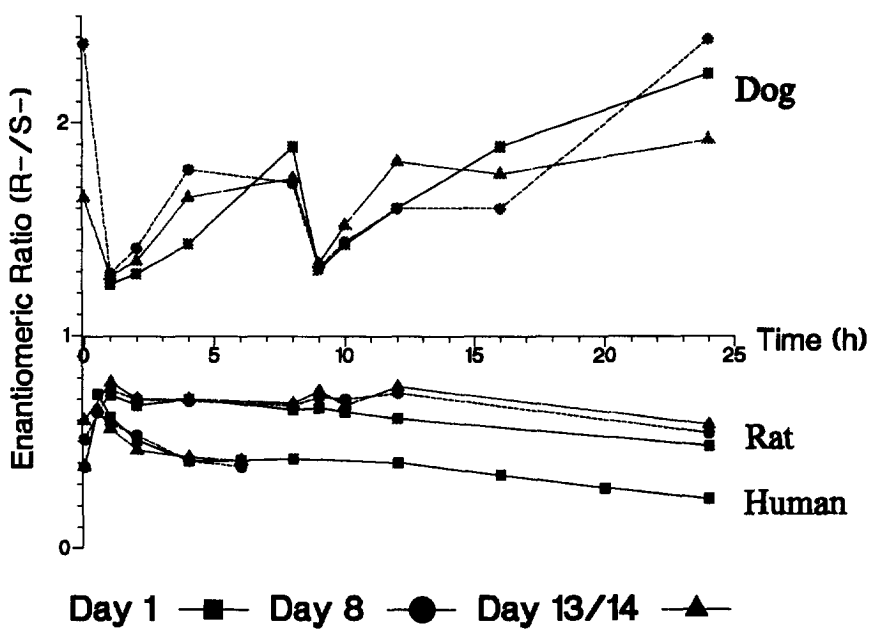


Fig. 7. Comparison of mean enantiomeric ratio of plasma concentrations (II/III) in the dog, rat and human after single and multiple dose administration (twice daily for rats and dogs and four times daily for man).

dog and human plasma. The specificity of the assay for each enantiomer was shown in all matrices. The method, as developed, has been proved to have sufficient sensitivity for accurate determination of the enantioselective pharmacokinetics of I in pre-clinical and clinical studies.

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