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Quantitation of 4-cyclohexyl-2-hydroxy-3-(3-methylsulfanyl-2-{2-[(morpholine-4-carbonyl)amino]-3-phenylpropionylamino}propionylamino)butyric acid isopropyl ester (CP-80,794), a renin inhibitor, and its hydrolytic cleavage metabolite 2-[(morpholine-4-carbonyl)amino]-3-phenylpropionic acid (CP-84,364) in dog and human plasma by high-performance liquid chromatography

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Abstract

Simple and precise high-performance liquid chromatographic (HPLC) assays were developed and validated for the determination of a renin inhibitor (RI), 4-cyclohexyl-2-hydroxy-3-(3-methylsulfanyl-2-{2-[(morpholine-4-carbonyl)amino]-3-phenylpropionylamino}propionylamino)butyric acid isopropyl ester (CP-80,794, I), and its hydrolytic cleavage metabolite, 2-[(morpholine-4-carbonyl)amino]-3-phenylpropionic acid (CP-84,364, II) in dog and human plasma. The internal standard for I, CP-83,092 (III, a carbon-substituted derivative of I) and analyte were extracted by liquid–liquid extraction using *n*-butyl chloride, cleaved to form a fragment containing a primary amine, and subsequently fluorescently derivatized for measurement by HPLC. Samples were analyzed by reversed-phase HPLC using a Waters C_{18} column with fluorescence detection at 390 nm excitation/440 nm emission. The quantitation limit of I was 10 ng/ml and the calibration curve was linear over the range of 0.01–1.0 μg/ml (r^2>0.99). In dog and human plasma, intra- and inter-assay precision ranged from 2.3 to 16% and 3.0 to 18%, respectively. The average recoveries were similar (≥63%) for both I and III and the upper limit of quantification of I can be as high as 4 μg/ml. The internal standard for II, CP-96,452 (IV, a methoxy derivative of II) and analyte were extracted by anion-exchange solid-phase extraction and subsequent liquid–liquid extraction using methyl *tert.*-butyl ether. Samples were analyzed by reversed-phase HPLC using a Waters C_{18} column with ultraviolet detection at 214 nm. The quantitation limit of II was 20 ng/ml and the calibration curve was linear over the range of 0.02–2.0 μg/ml (r^2>0.99). In dog and human plasma, intra- and inter-assay precision ranged from 2.6 to 13.0% and 1.8 to 20.0%, respectively. The average recoveries were similar (≥75%) for both II and IV and the upper limit of quantification of II can be as high as 20 μg/ml. The methods described have been successfully applied to the quantification of I and II in about 5000 dog and human plasma samples over a 2 year period. © 1997 Published by Elsevier Science B.V.

Keywords: 4-Cyclohexyl-2-hydroxy-3-(3-methylsulfanyl-2-{2-[(morpholine-4-carbonyl)amino]-3-phenylpropionylamino}propionylamino)butyric acid isopropyl ester; 2-[(Morpholine-4-carbonyl)amino]-3-phenylpropionic acid

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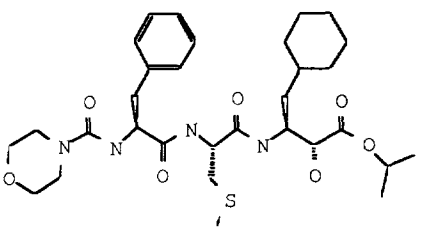
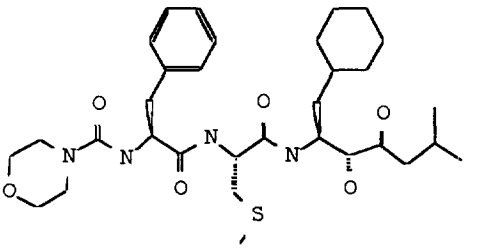
1. Introduction

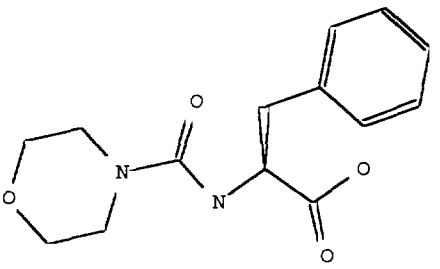
Inhibition of the renin-angiotensin system (RAS) is indicated for hypertension and congestive heart failure (CHF). There are presently three methods of inhibiting the RAS; renin inhibition (RI), angiotensin-converting enzyme inhibition (ACE-I) and angiotensin II (AII) receptor antagonism. CP-80,794 (4-cyclohexyl-2-hydroxy - 3 - (3 - methylsulfanyl-2-{2-[(morpholine-4-carbonyl)amino]- 3 -phenylpropionylamino}propionylamino)butyric acid isopropyl ester, I) is an orally active Pfizer RI shown to lower blood pressure and plasma renin activity (PRA) in sodium-deficient cynomolgus and marmoset monkeys [1,2]. To aid in the development of this RI and facilitate studies of the pharmacokinetics of I in dogs and humans, we developed a sensitive and selective method for the determination of the drug in dog and human plasma. Following initial oral dosing studies with I, a circulating hydrolytically cleaved metabolite was identified (CP-84,364, II), and a method for its determination was subsequently developed. The HPLC assay for 4 - cyclohexyl - 2 - hydroxy -3 - (3 - methylsulfanyl - 2 - {2-[(morpholine - 4- carbonyl)-amino] - 3 - phenylpropionylamino}propionylamino)-butyric acid isopropyl ester (CP-80,794, I, Fig. 1) was validated and characterized over the range of 0.01–1 μg I/ml of plasma. The UV–HPLC assay for 2-[(morpholine - 4 - carbonyl)amino] - 3 - phenylpropionic acid (CP-84,364, II, Fig. 1) was validated and characterized over the range of 0.02–2 μg II/ml plasma.



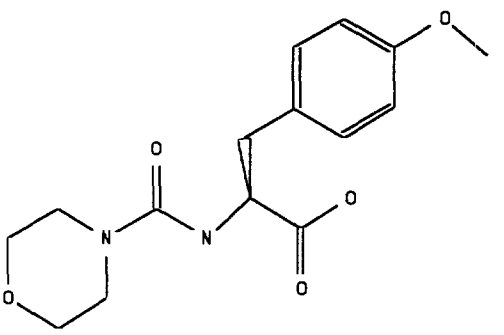
CP-80,794, I



CP-83,092, III



CP-84,364, II



CP-96,452, IV

Fig. 1. Structures of CP-80,794 (I) and CP-84,364 (II), and their respective internal standards, CP-83,092 (III) and CP-96,452 (IV).

2. Experimental

2.1. Compounds I and III

2.1.1. Materials

CP-80,794 (I) and CP-83,092 (III), a carbon-substituted derivative of I used as an internal standard, were synthesized at Pfizer Central Research (Groton, CT, USA) (Fig. 1). HPLC-grade *n*-butyl chloride was purchased from Burdick and Jackson (Muskegon, MI, USA). HPLC-grade acetonitrile (CH_3CN) and reagent grade ethanol (EtOH), potassium phosphate monobasic (KH_2PO_4) and glycine were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Solid sodium hydroxide (NaOH) was obtained from J.T. Baker (Phillipsburg, NJ, USA). *o*-Phthalaldehyde (Fluoraldehyde, OPA) was purchased from Pierce (Rockford, IL, USA), Hydroxypropyl-beta-cyclodextrin (HPBCD) was purchased from Research Biochemicals International (Natick, MA, USA), Fluorescamine (Fluram) was purchased from Roche Diagnostics (Palo Alto, CA, USA), and chymotrypsin was purchased from Sigma (St. Louis, MO, USA). The water used for preparing mobile phase and aqueous solutions was obtained from a Millipore Milli-EQ System (Bedford, MA, USA), 18.2 MΩ.

2.1.2. Equipment

A Perkin-Elmer ISS-100 (Perkin-Elmer, Norwalk, CT, USA) automated injector was used to deliver a 75 μl volume onto a LDC/Milton Roy (Riviera Beach, FL, USA) liquid chromatography system. A Kratos Spectroflow 980 programmable fluorescence detector with a 440 nm emission filter was used to monitor fluorescence. A Novapak C_{18} column (150× 3.9 mm, 5 μm) from Waters (Milford, MA, USA) with a 0.5 μm filter and a C_{18} 40 μm pre-column was used. Peak heights of I and III were measured by a Spectra Physics (Stockholm, Sweden) SP4200 computing integrator.

2.1.3. Chromatographic conditions

The flow-rate was set to 1 ml/min and effluent was monitored at 390 nm excitation and 440 nm emission. The HPLC mobile phase consisted of water–CH_3CN (73:27, v/v). All analyses were performed at ambient temperature.

2.1.4. Sample preparation

After addition of 100 μl of a 2 μg/ml internal standard solution (III), a 1 ml aliquot of plasma was basified to pH 12 with 100 μl of 1 *M* NaOH. The samples were extracted with 5 ml *n*-butyl chloride by shaking for 10 min at 100 rpm. After centrifugation, the organic layer was transferred into a disposable conical centrifuge tube containing 2 ml of CH_3CN and evaporated to dryness. To the dried residue, 20 μl of an *o*-phthalaldehyde (OPA) and 5% HPBCD solution (i.e., 1.5 mg OPA dissolved in 150 μl EtOH+150 mg HPBCD+2.85 ml 0.1 *M* potassium phosphate buffer, pH 7.9) was added, samples vortexed for 30 s and incubated at ambient temperature for 45 min. To eliminate excess OPA, 10 μl of a glycine and 5% HPBCD solution (i.e., 3 mg glycine+50 mg HPBCD+1 ml 0.1 *M* potassium phosphate buffer, pH 7.9) was added, samples vortexed for 30 s and incubated at ambient temperature for 15 min. To cleave the intact CP-80,794 to the amine fragment, 10 μl of a solution containing 500 n*M* chymotrypsin and 5% HPBCD (i.e., 1 mg chymotrypsin+50 mg HPBCD+1 ml 0.1 *M* potassium phosphate buffer, pH 7.9) was added, samples vortexed for 30 s and incubated at 37°C for 15 min. The newly formed amine fragment was then derivatized with 50 μl of an acetonitrile solution containing 90 n*M* fluorescamine. A 75 μl aliquot was then injected onto the HPLC system for analysis. All derivatizations were optimized for the greatest yield at the times and temperatures described above.

2.2. Compounds II and IV

2.2.1. Materials

CP-84,364 (II) and CP-96,452 (IV), a *p*-methoxy derivative internal standard, were synthesized at Pfizer Central Research (Fig. 1). HPLC-grade methyl *tert.*-butyl ether (MTBE) was purchased from Burdick and Jackson. HPLC-grade CH_3CN, methanol (MeOH) and KH_2PO_4 were obtained from Fisher Scientific and 37% hydrochloric acid (HCl) and solid NaOH were obtained from J.T. Baker. The water used for preparing mobile phase and aqueous solu-

tions was obtained from a Millipore Milli-EQ System, 18.2 MΩ.

2.2.2. Equipment

The HPLC system consisted of a Waters M6000A solvent delivery system and an ISS-100 autosampler (Perkin-Elmer) connected to a LDC/Milton Roy SM4000 variable-wavelength spectrophotometer. A Novapak C_{18} column (75×3.9 mm I.D., 5 μm) from Waters was used for the HPLC separations. II and IV peak heights were measured by a Spectra Physics SP4200 computing integrator.

2.2.3. Chromatographic conditions

The flow-rate was set to 1 ml/min and effluent was monitored at 214 nm. The mobile phase composition was 100 m*M* KH_2PO_4 (pH 2.3)–acetonitrile (86:14, v/v). All analyses were performed at ambient temperature.

2.2.4. Sample preparation

After addition of 50 μl of a 10 μg/ml internal standard solution (IV), a 0.5 ml aliquot of plasma was basified with 1 ml of 0.1 *M* NaOH. The samples were loaded onto a Varian (Harbor City, CA, USA) anion-exchange Bond Elute column (SAX, 5 cm^3/500 mg) conditioned with 1 ml of MeOH and 1 ml of distilled/deionized water. The column was then washed with 1 ml distilled/deionized water, and sample eluted with 2×1 ml 0.1 *M* KH_2PO4 (pH 3). After elution, the sample was acidified with 25 μl of 1 *M* HCl and extracted with 5 ml MTBE by shaking for 10 min at 100 rpm. After centrifugation for 5 min at 2000 *g*, the organic solvent was transferred to clean tubes and evaporated to dryness. The samples were then reconstituted in mobile phase and transferred to injection vials. A 100 μl aliquot out of a total of 125 μl was then injected onto the HPLC system for analysis.

2.3. Quantification of I and II

Calibration standards were prepared by adding a known amount of I and III or II and IV to 1 or 0.5 ml of plasma for quantification of I or II, respectively. Standard curves for I in dog plasma consisted of solutions at concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 μg/ml. Standard curves for I in human plasma consisted of solutions at concentrations of 0.01, 0.025, 0.05 and 0.1 μg/ml. Standard curves for II in dog plasma consisted of solutions at concentrations of 0.05, 0.1, 0.2, 0.5, 1 and 2 μg/ml. Standard curves for II in human plasma consisted of solutions at concentrations of 0.02, 0.05, 0.1 and 0.2 μg/ml. The sample extraction and HPLC analyses were carried out as described for I and II in Section 2.1 Section 2.2. A least-squares linear regression fit was made of known analyte concentrations to SP4200 measured I or II to internal standard peak height ratios. From the standard curve the unknown drug or metabolite concentrations were calculated. A weighting factor of $1/x$ was applied in the regression analysis.

3. Results and discussion

3.1. Selectivity

Representative chromatograms of the analytes in dog and human plasma are shown in Figs. 2 and 3, respectively. No chromatographic peaks in the window necessary for analysis interfered with the detection of I, II, III or IV in dog or human plasma. Under the chromatographic conditions described, the retention times of these compounds were approximately 4, 11, 6 and 13 min, respectively.

3.2. Recovery of I and II from dog and human plasma

Compound I recovery samples were analyzed at 0.1 μg/ml. The recovery was determined by comparing peak heights from four unextracted standards with those of four extracted standards at the same concentration. Mean% recovery (±S.D.) for I in dog and human plasma was 90% (±3.1) and 75% (±1.2). Recovery of II was monitored at 1 μg/ml (n=4) and determined to be 63% (±4.2) from dog and 71% (±2.3) from human plasma.

3.3. Assay characterization for I

The lower limit of quantitation for the assay was set at 10 ng/ml with a relative standard deviation of

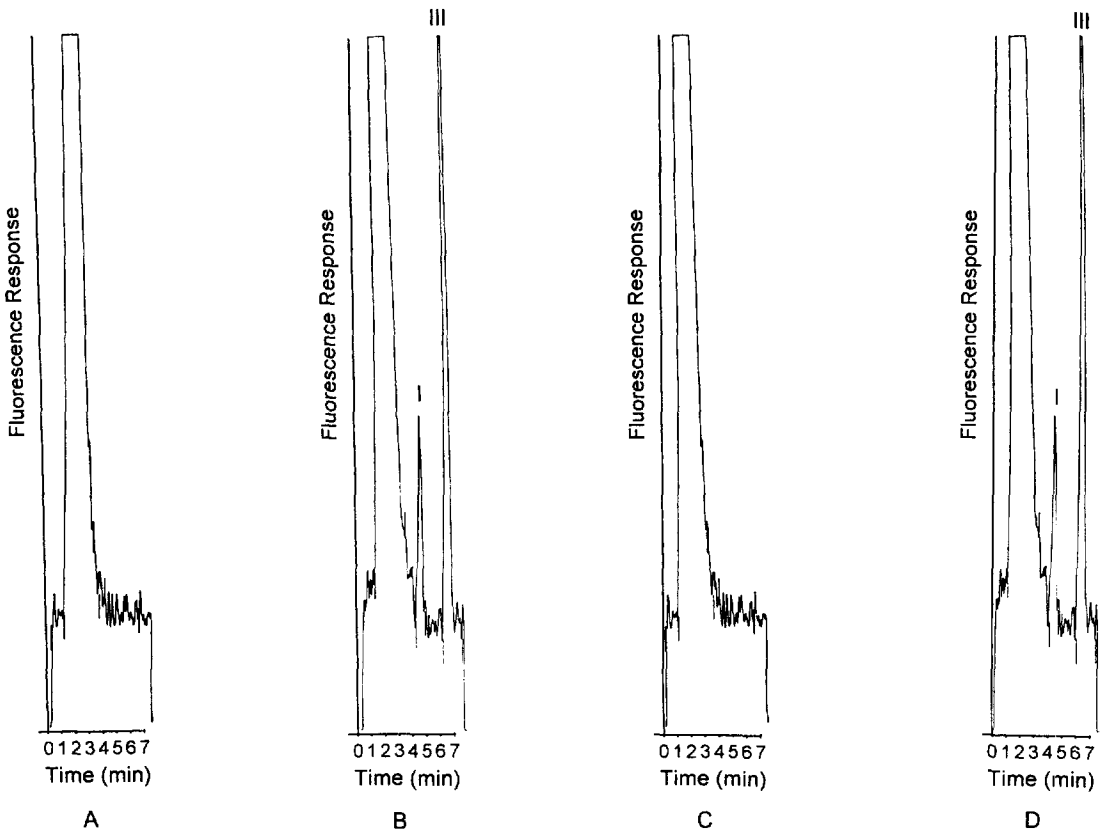


Fig. 2. Representative chromatograms of I (CP-80,794) and III (CP-83,092, internal standard for I) in dog and human plasma. (A) Drug-free control dog plasma extract, (B) control dog plasma extract containing 0.05 μg/ml I and 200 ng III, (C) drug-free control human plasma extract and (D) control human plasma containing 0.05 μg/ml I and 200 ng III.

less than 20%. A summary of the intra-assay and inter-assay validation data in dog and human plasma is presented in Tables 1 and 2, respectively. The assays exhibited linearity (r^2>0.99) over the 0.01–1 μg/ml range in plasma.

3.4. Assay characterization for II

Intra- and inter-assay accuracy and precision data are summarized in Tables 3 and 4, respectively. The lower and upper limits of assay quantitation were set at 0.02 and 0.2 μg/ml, respectively for human plasma and 0.05 and 2 μg/ml, respectively for dog plasma. The assays exhibited linearity (r^2>0.99) over the 0.02–2 μg/ml range in plasma.

4. Conclusions

Simple and reproducible procedures for the quantification of a renin inhibitor and its circulating metabolite in dog and human plasma have been developed. The use of chemical derivatization is documented to be an effective means of improving the detectability of a compound by introducing functional groups that interact strongly with a particular detector [3]. For compound I, fluorescent derivatization enabled the use of fluorescence detection and results in increased sensitivity. The methods described were successfully applied to toxicokinetic studies in dogs and clinical toleration studies in man. Fig. 4 shows plasma concentration versus time profiles of I and II following a 400

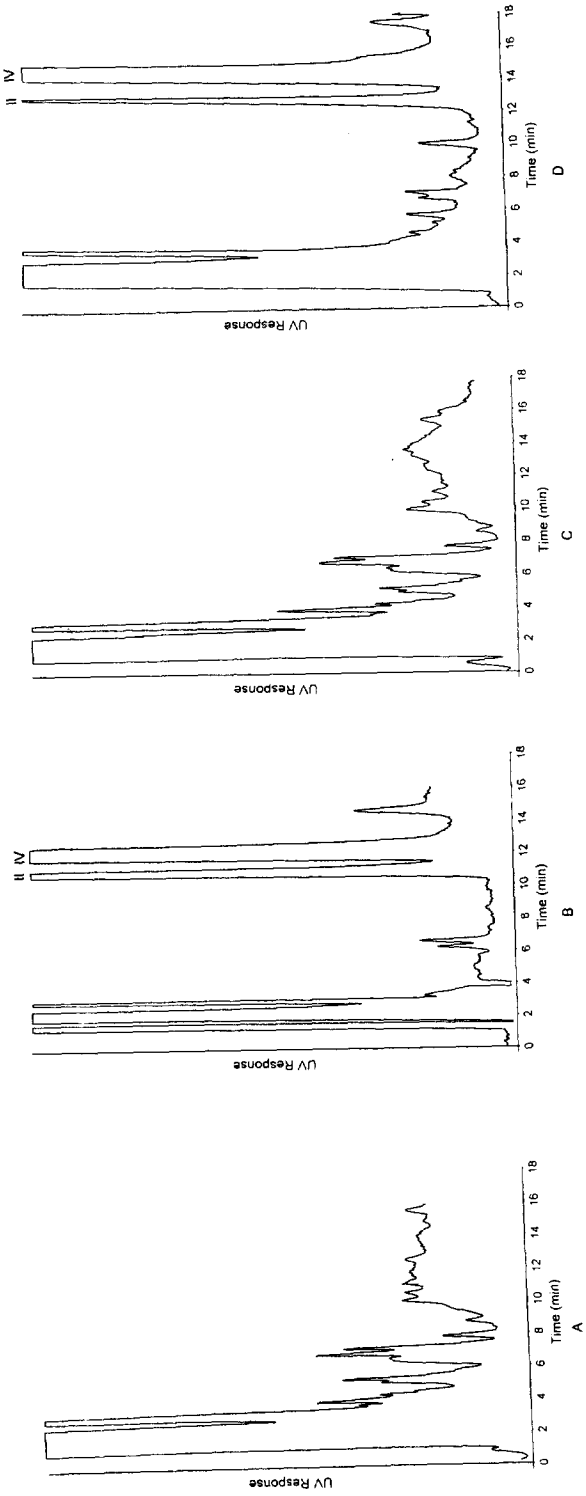


Fig. 3. Representative chromatograms of II (CP-84,364) and IV (CP-96,452, internal standard for II) in dog and human plasma. (A) Drug-free control dog plasma extract, (B) control dog plasma extract containing 0.2 μg/ml II and 500 ng IV, (C) drug-free control human plasma extract and (D) control human plasma containing 0.2 μg/ml II and 500 ng IV.

Table 1
Intra-day accuracy and precision values for the determination of CP-80,794 (I) in dog and human plasma

Plasma species	Concentration (μg/ml)	Mean (μg/ml)	Standard deviation	Accuracy (%)	Precision (%)
Dog	0.01	0.010	0.001	100	10
	0.025	0.025	0.004	100	16
	0.05	0.048	0.002	96	4
	0.1	0.101	0.003	101	3
	0.25	0.247	0.010	99	4
	0.5	0.508	0.025	102	5
	1	0.998	0.023	100	2
Human	0.01	0.011	0.001	110	9
	0.025	0.022	0.002	88	9
	0.05	0.052	0.002	104	4
	0.1	0.100	0.008	100	8

$n=5$.

Table 2
Inter-day accuracy and precision values for the determination of CP-80,794 (I) in dog and human plasma

Plasma type	Concentration (μg/ml)	Mean (μg/ml)	Standard deviation	Accuracy (%)	Precision (%)
Dog	0.01	0.011	0.002	110	18
	0.025	0.027	0.003	108	11
	0.05	0.049	0.002	98	4
	0.1	0.101	0.005	101	5
	0.25	0.251	0.012	100	5
	0.5	0.511	0.027	102	5
	1	0.999	0.025	100	3
Human	0.01	0.011	0.002	110	18
	0.025	0.024	0.003	96	13
	0.05	0.049	0.003	98	6
	0.1	0.104	0.002	104	2

$n=3$; three separate assay days.

Table 3
Intra-day accuracy and precision values for the determination of CP-84,364 (II) in dog and human plasma

Plasma type	Concentration (μg/ml)	Mean (μg/ml)	Standard deviation	Accuracy (%)	Precision (%)
Dog	0.05	0.058	0.005	116	9
	0.10	0.109	0.010	109	9
	0.20	0.196	0.004	98	2
	0.50	0.468	0.062	94	13
	1.00	1.024	0.046	102	4
	2.00	1.996	0.052	100	3
Human	0.02	0.024	0.002	120	8
	0.05	0.049	0.004	98	8
	0.1	0.094	0.003	94	3
	0.2	0.202	0.006	101	3

$n=4$.

Table 4
Inter-day accuracy and precision values for the determination of CP-84,364 (II) in dog and human plasma

Plasma type	Concentration (μg/ml)	Mean (μg/ml)	Standard deviation	Accuracy (%)	Precision (%)
Dog	0.05	0.056	0.003	112	5
	0.10	0.106	0.007	106	7
	0.20	0.199	0.005	100	3
	0.50	0.479	0.032	96	7
	1.00	1.011	0.020	101	2
	2.00	1.995	0.050	100	3
Human	0.02	0.020	0.004	100	20
	0.05	0.049	0.002	98	4
	0.1	0.099	0.008	99	8
	0.2	0.201	0.004	101	2

$n=3$; three separate assay days.

mg/kg dose to a representative dog. Fig. 5 illustrates human plasma concentration versus time profiles of I and II following a 100 mg CP-80,794 single oral dose. The methods described were used in the quantification of I and II in about 5000 dog and human plasma samples over a 2 year period.

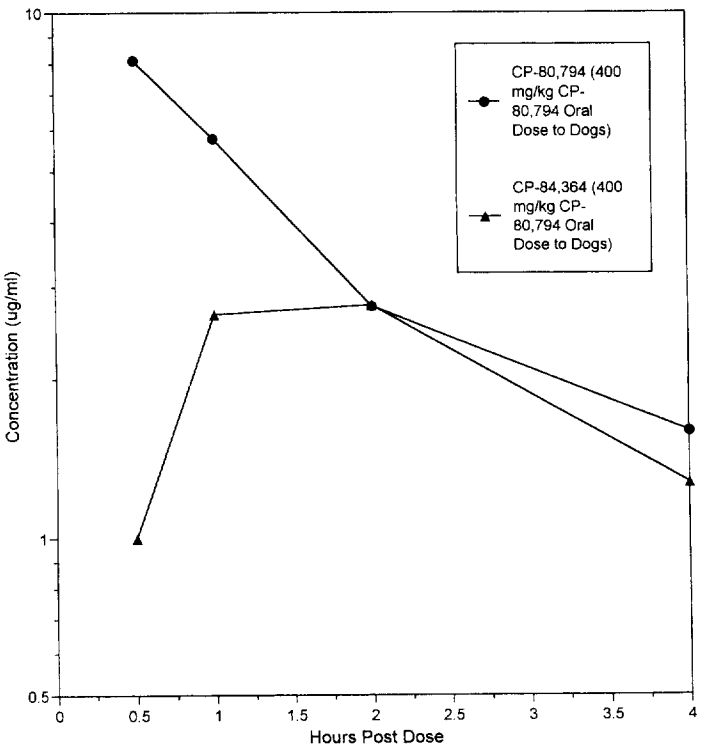


Fig. 4. Plasma concentrations of I (●) and II (▲) following a single 400 mg/kg oral dose of I to a representative dog.

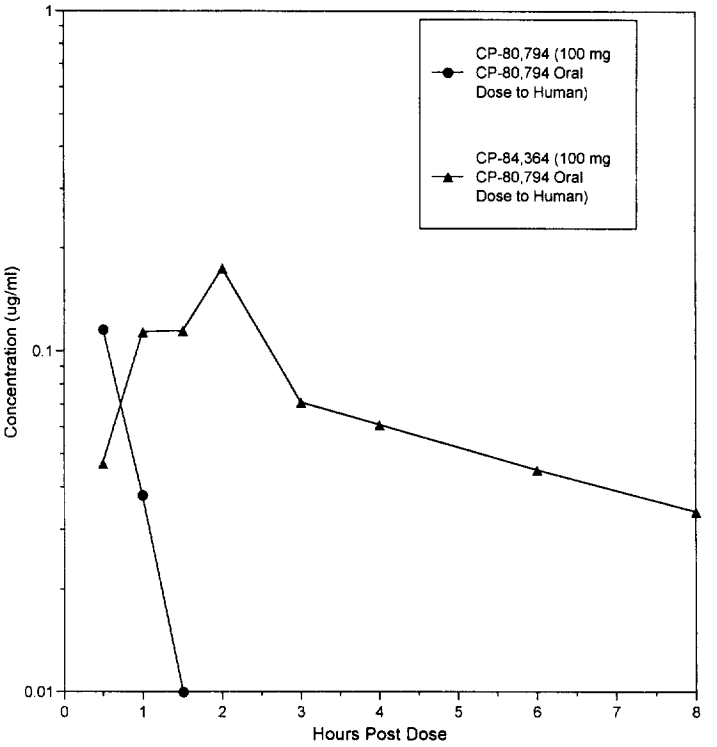


Fig. 5. Plasma concentrations of I (●) and II (▲) following a single 100 mg oral dose of I to a representative human.

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