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High-performance liquid chromatographic assay for CI-1004, a dual-inhibitor antiinflammatory agent, in rat, rabbit, dog, monkey and human plasma

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Abstract

CI-1004 and PD 138389 (internal standard, I.S.), were isolated from rat, rabbit, dog, monkey and human plasma by solid-phase extraction with Bond-Elut C18 cartridges. Liquid chromatographic separation was achieved isocratically on a Zorbax Rx-C8 analytical column (250 mm×4.6 mm I.D). The mobile phase consisted of acetonitrile–20 m*M* ammonium acetate (65:35, v/v), (pH 4.0). Column temperature was either 40°C (human assay) or 45°C and column effluent was monitored spectrophotometrically at 360 nm. Specificity, chromatographic performance parameters, system repeatability, recovery from matrix, linearity, precision, accuracy and stability were evaluated. Mean retention times (±S.D.) of CI-1004 and I.S. were 7.8±0.1 and 10.9 ±0.2 at 40°C and 7.7±0.2 min and 10.7±0.2 min at 45°C. No interfering peaks were observed at the retention time of CI-1004 throughout the validation process. Peak height ratios were proportional to CI-1004 over the concentration range of 7.5–5000 ng/ml in rat, rabbit and monkey plasma and 2.5–5000 ng/ml in dog and human plasma. Recovery of low, medium and high standards of CI-1004 ranged from 82.8–107% from all animal species and recovery of I.S. from rat, rabbit, dog and monkey plasma ranged from 77.5–82.0% and from human plasma was 111%. Assay precision for CI-1004 based on quality control samples was less than or equal to 8.5% C.V. with an accuracy (percentage relative error) of ±4.7% for all species. Minimum quantitation limit of CI-1004 was 7.5 ng/ml for 0.2 ml rat, rabbit and monkey plasma samples and 2.5 ng/ml for 0.5 ml dog and human plasma samples. The method is suitable for studying the preclinical and clinical pharmacokinetics of CI-1004; © 1997 Elsevier Science B.V.

Keywords: CI-1004

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammatory conditions such as rheumatoid and osteoarthritis. At sites of inflammation, arachidonic acid is converted to biologically active mediators through the activities of two categories of enzymes. Cyclooxygenases catalyze the first and rate-limiting step in the production of the prostaglandin family of mediators. Lipoxygenases, particularly 5-lipoxygenase, catalyze the formation of lipid hydroperoxides which may be further converted to leukotrines. Compounds which inhibit both cyclooxygenase and 5-lipoxygenase (dual inhibitors) may prove to be more potent anti-inflammatory

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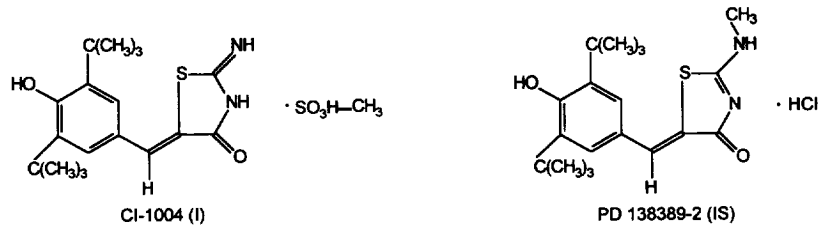

Fig. 1. Structures of I and the I.S.

agents with a reduced risk for gastrointestinal pathology.

CI-1004 (I), 4-thiazolidinone, 5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl] methylene]-2-imino-, (Z)-, methanesulfonate (1:1) salt, is a potent cyclooxygenase and 5-lipoxygenase inhibitor being developed for use in treating rheumatoid and osteoarthritis. Due to its potency, doses of 1–10 mg/kg were used in preclinical pharmacokinetic studies and an initial dose of 1 mg was used in a rising dose tolerance clinical trial. A selective and sensitive method was needed to characterize the concentration–time profiles of I in plasma after these low doses. This report describes the development and validation of a liquid chromatographic assay for I in rat, rabbit, dog, monkey and human plasma. The structures of I and the internal standard, PD 138389-2 (I.S.), are shown in Fig. 1.

2. Materials and methods

2.1. Chemicals

All chemicals were HPLC grade or reagent-grade unless noted otherwise. Acetonitrile was obtained from Mallinckrodt (Paris, KY, USA). Water, methanol and ammonium hydroxide were purchased from EM Science (Gibbstown, NJ, USA). Ammonium acetate was purchased from Aldrich (Milwaukee, WI, USA). Glacial acetic acid was purchased from Baker (Phillipsburg, NJ, USA). Heparinized rat, dog and monkey plasma was obtained from Pel-Freez Biologicals (Rogers, AR, USA). Heparinized rabbit plasma was obtained from Parke-Davis Pharmaceutical Research, Division of Warner–Lambert (Ann Arbor, MI, USA) in-house colony. Heparinized human plasma was purchased from Interstate Blood Bank (Memphis, TN, USA). Bond-Elut C18 cartridges (100 mg sorbent, 1.0 ml cartridge volume) were purchased from Analytichem International (Harbor City, CA, USA). Twenty-four port vacuum manifold was obtained from Alltech Associates (Deerfield, IL, USA). I and I.S. were obtained from Parke-Davis.

2.2. Chromatographic equipment and conditions

The HPLC system consisted of a SP 8800 solvent delivery system from Spectra Physics (San Jose, CA, USA), an ISS-200 automated sample injector from Perkin–Elmer (Norwalk, CT, USA), a Spectroflow 783 UV–Vis detector from Applied Biosystems (Ramsey, NJ, USA), a ChromJet integrator from Spectra Physics and TCM column oven from Waters Chromatography Division (Milford, MA, USA). Zorbax Rx-C8 guard column, 12.5×4.0 mm I.D., 5-μm particle size and Zorbax Rx-C8 analytical column, 250×4.6 mm I.D., 5-μm particle size were purchased from MacMod Analytical (Chadds Ford, PA, USA). Mobile phase buffer was 20 m*M* ammonium acetate adjusted to pH 4.0 with glacial acetic acid. Mobile phase acetonitrile–buffer ratio was 65:35 (v/v) with a flow-rate of 1.0 ml/min. The column was maintained at 45°C for rat, rabbit, dog and monkey assays and 40°C for the human assay. Column effluent was monitored at a wavelength of 360 nm. Sample injection volume was 100 μl of sample extraction eluant.

2.3. Standards

A 1 mg/ml stock solution of I was prepared in methanol every 48 h and stored at −20°C. The stock solution was serially diluted with plasma to prepare working solution A (100 000 ng/ml) and working solution B (10 000 ng/ml). Working solutions were stored in single-use aliquots at −20°C for up to 3

months. Calibration standards were prepared on the assay day by serially diluting working standard A or B with plasma to make a calibration curve ranging from 7.5–5000 ng/ml (10 levels) for rat, rabbit and monkey plasma assays and 2.5–5000 ng/ml (11 levels) for dog and human plasma assays. A 1 mg/ml stock solution of I.S. was prepared in methanol every 2 weeks and stored at −20°C. A 5000 ng/ml working solution in methanol was prepared on the assay day from the stock solution.

2.4. Sample preparation

Frozen samples were thawed at room temperature for a minimum of 30 min, vortexed for 30 s then centrifuged for 10 min at 2800 *g*. A 50-μl aliquot of the I.S. working solution was added to each 0.2 ml rat, rabbit and monkey sample and a 100-μl aliquot was added to each 0.5 ml dog and human samples. All but human plasma samples were diluted with 0.5 ml 0.5% NH_4OH and vortexed immediately; human plasma samples were diluted with 0.25 ml 0.5% NH_4OH. C18 cartridges were conditioned with two 1-ml volumes of methanol followed with a 1-ml volume of water. Vacuum pressure was maintained at approximately 10–20 kPa, unless otherwise noted. The entire sample was applied to the cartridge, followed by a 1-ml water wash that had been used to first rinse the sample tube. The cartridge was washed with 1 ml of 25% acetonitrile. Cartridges were not permitted to dry until this step. Cartridges were dried for 1 min at 40–60 kPa. The manifold was removed and probes wiped with absorbent tissue and collection tubes inserted. Compounds were eluted with a 0.5-ml aliquot of 65% acetonitrile for all but the human plasma assay; the human assay required three 0.25-ml aliquot additions of the elution solvent. The cartridges were dried for 15 s at 30–40 kPa after each addition of elution solvent. The eluate was centrifuged for 15 min at 2800 *g* prior to transferring to injection vials.

2.5. Data collection and calculation

Calibration curves for rat, rabbit, dog and monkey plasma were characterized by assaying each calibration standard in triplicate on three separate occasions. Calibration curves for human plasma were characterized by assaying each calibration standard singly on three separate occasions. Linear regression analysis of calibration standard data was performed by regressing peak-height ratios (I/I.S.) on concentrations of drug in plasma. Slopes, intercepts and coefficients of determination were determined and evaluated for linearity and reproducibility. During analysis of preclinical and clinical study samples, all calibration curves were assayed singly.

2.6. Accuracy and precision

Quality control (QC) samples were prepared in rat (30, 300 and 2400 ng/ml), rabbit (15, 300 and 2400 ng/ml), dog (6, 120 and 2400 ng/ml), monkey (15, 300 and 2400 ng/ml) and human (7.8, 125 and 2000 ng/ml) plasma and stored at −20°C. Each concentration was prepared in a manner to assure a greater than 95% biological matrix volume. During assay validation, QC samples were analyzed in triplicate on three separate occasions. Accuracy was expressed as relative error (%R.E.), of the percent deviation of the mean ($n=9$) observed concentration from the theoretical value. Precision was expressed as the coefficient of variation of observed concentrations, expressed as a percentage (%C.V.). QC samples were also assayed in duplicate in each batch during preclinical and clinical study sample analysis and used as indicators of assay validity and sample stability.

2.7. Lower limit of quantitation

The lower limit of quantification (LLOQ) was determined by evaluating samples prepared in rat plasma at the nominal concentration of 7.5 ng/ml and in human plasma at 2.5 ng/ml. The samples were prepared in a manner so that the content of biological matrix was 95% or greater. During the validation process the LLOQ samples were analyzed in triplicate on two separate occasions and deemed acceptable if accuracy and precision were within 20%.

2.8. Selectivity

Selectivity was assessed by the lack of interfering substances eluting at the same retention time as I or I.S. In addition, selectivity was further assessed by rapid-scanning photodiode array detection (Waters

991 photodiode array detector). Overlays of UV spectra were evaluated at the ascending, apex and descending portions of I and I.S. peaks from a solution standard, a QC sample and plasma samples collected after dosing I.

2.9. Recovery

Recovery of I from plasma was determined at concentrations of 30, 300 and 2400 ng/ml in rat and dog plasma, 30, 300 and 3000 ng/ml in rabbit plasma, 10, 100 and 1000 ng/ml in monkey plasma and 8, 200 and 2000 ng/ml in human plasma. Recovery of the I.S. was determined at 1250 ng/ml in rat, rabbit and monkey plasma and 1000 ng/ml in dog and human plasma. Peak areas of I and I.S. obtained from extracted plasma samples were compared with peak areas of standards prepared in elution solvent and injected directly.

2.10. System repeatability

System repeatability was determined by injection of nine replicates of extracted plasma samples at low and high concentrations of I and typical I.S. concentrations. System repeatability was expressed as the percentage C.V. of mean peak heights.

2.11. Stability

The stability of I and I.S. was evaluated in an injection solvent (CH_3CN–H_2O, 65:35, v/v). Initial peak areas of standards at known concentrations of I and I.S. were determined. Additional aliquots of I and I.S. in injection solvent were stored at room temperature and reassayed over 6 days. Stability was evaluated by comparing initial and subsequent peak areas.

The stability of I was also evaluated in plasma at room temperature. Initial peak response of extracted plasma samples were determined. Additional plasma aliquots were stored at room temperature and reassayed over a 24-h period. Stability was evaluated by comparing peak response from initial and subsequent determinations.

Stability was further assessed by subjecting I in plasma to three freeze–thaw cycles. Samples were analyzed after the third freeze–thaw cycle and peak response was compared to mean peak response obtained from non-frozen samples analyzed immediately after preparation.

2.12. Applicability of the methods

The suitability of the methods for investigating I pharmacokinetics was assessed by analyzing plasma following oral administration of I at 1.1 mg/kg to male Wistar rats, 10 mg/kg to female New Zealand White rabbits, 1 mg/kg to male beagle dogs and 10 mg to a human subject. Heparinized blood samples were obtained prior to and serially at selected time points after dosing. Plasma was harvested by centrifugation and stored at −20°C until analysis.

3. Results

3.1. Data collection and calculation

Peak height ratios of calibration standards were proportional to the concentration of I in plasma of all species over the ranges tested. Calibration curves were linear and well described by least squares regression lines using a weighting factor of 1/concentration^2 [1] to achieve homogeneity of variance (Table 1). Mean calculated concentrations of the

Table 1
Mean (n=3) linear regression parameters for I calibration curves in rat, rabbit, dog, monkey and human plasma over 3 batch runs during assay validation

Species	Slope (±S.D.)	Intercept (±S.D.)	Correlation coefficient (±S.D.)
Rat	0.150 (0.0087)	−0.0135 (0.165)	0.998 (0.0010)
Rabbit	0.139 (0.0070)	−0.0085 (0.030)	1.000 (0.00001)
Dog	0.175 (0.0074)	0.0513 (0.0128)	0.999 (0.0006)
Monkey	0.153 (0.0040)	0.0645 (0.0648)	0.999 (0.0010)
Human	0.193 (0.0100)	0.145 (0.0304)	0.999 (0.0004)

calibration standards were within −4.2–3.2% of theoretical concentrations across all assays.

3.2. Selectivity

The assays were adequately selective for I and I.S. in all species. Retention times of I and I.S. in animal plasma were 7.7±0.2 and 10.7±0.2 min and in human plasma were 7.8±0.1 and 10.9±0.2 min, respectively. Chromatograms of extracts obtained from 6 or more lots of blank plasma from each species were free of peaks at the retention time of I and I.S.. Representative chromatograms of extracted blank plasma, a 100 ng/ml I calibration standard and sample chromatograms after an oral dose of I are shown in Fig. 2.

System specificity was further determined using rapid-scanning photodiode array detection. The spectral overlays of the ascending, apex and descending portions of both I and I.S. peaks from a solution standard, a QC sample and a post-dose plasma sample were virtually superimposable.

3.3. System repeatability

System repeatability was less than ±4.2% and ±1.3% for I, at low and high concentrations, respectively. System repeatability was less than ±1.8% (%C.V.) for I.S. at typical concentrations.

3.4. Recovery

Recovery of I was high for all species at all concentrations and ranged from 83–107% (Table 2). Recovery of I.S. was approximately 80% from all plasma sources at typical concentrations used during analysis (Table 2).

3.5. Stability of stock solutions

Peak response of stock solutions of I stored for 48 h at ambient temperature and −20°C were within 10% of that on the day of preparation. Peak response of stock solutions of I.S. stored for 2 weeks at −20°C were within 10% of that on the day of preparation.

3.6. Stability in injection solvent

The peak response of I and I.S. in injection solvent after 6 days at room temperature were 100.4% and 100.9% of initial response, respectively, indicating stability for at least this time period.

3.7. Stability in plasma

Concentrations of I QC samples stored at −20°C were within 10% of validated values for 10 months in rat plasma, at least 10 months in rabbit plasma, 13 months in dog plasma, 18 months in monkey plasma and at least 12 months in human plasma. Peak response of I in rat, dog, monkey and human plasma after 24 h at ambient temperature was within 5% of controls processed immediately and within 6% after 7 h at 37°C. Stability of I in plasma after subjecting triplicate standards to three freeze–thaw cycles were within ±10% of mean control values obtained from non-frozen samples for all species.

3.8. Accuracy and precision

Assay precision and accuracy were determined by assaying 3 quality control samples in triplicate in 3 separate batch runs. Intra-run assay precision (%C.V.) and accuracy (%R.E.) was 15.3% or less and ±9.5%, respectively, for all five species (Table 3). Respective inter-run values were 8.3% or less and ±4.7% for all five species (Table 3).

3.9. Lower limit of quantitation

The lower limit of quantitation, based on acceptable accuracy and precision, was 7.5 ng/ml for 0.2-ml plasma samples and 2.5 ng/ml for 0.5-ml samples.

3.10. Applicability of the methods

Representative concentration–time profiles following oral administration of I to male Wistar rats, female New Zealand White rabbits, male beagle dogs and a human subject are depicted in Fig. 3.

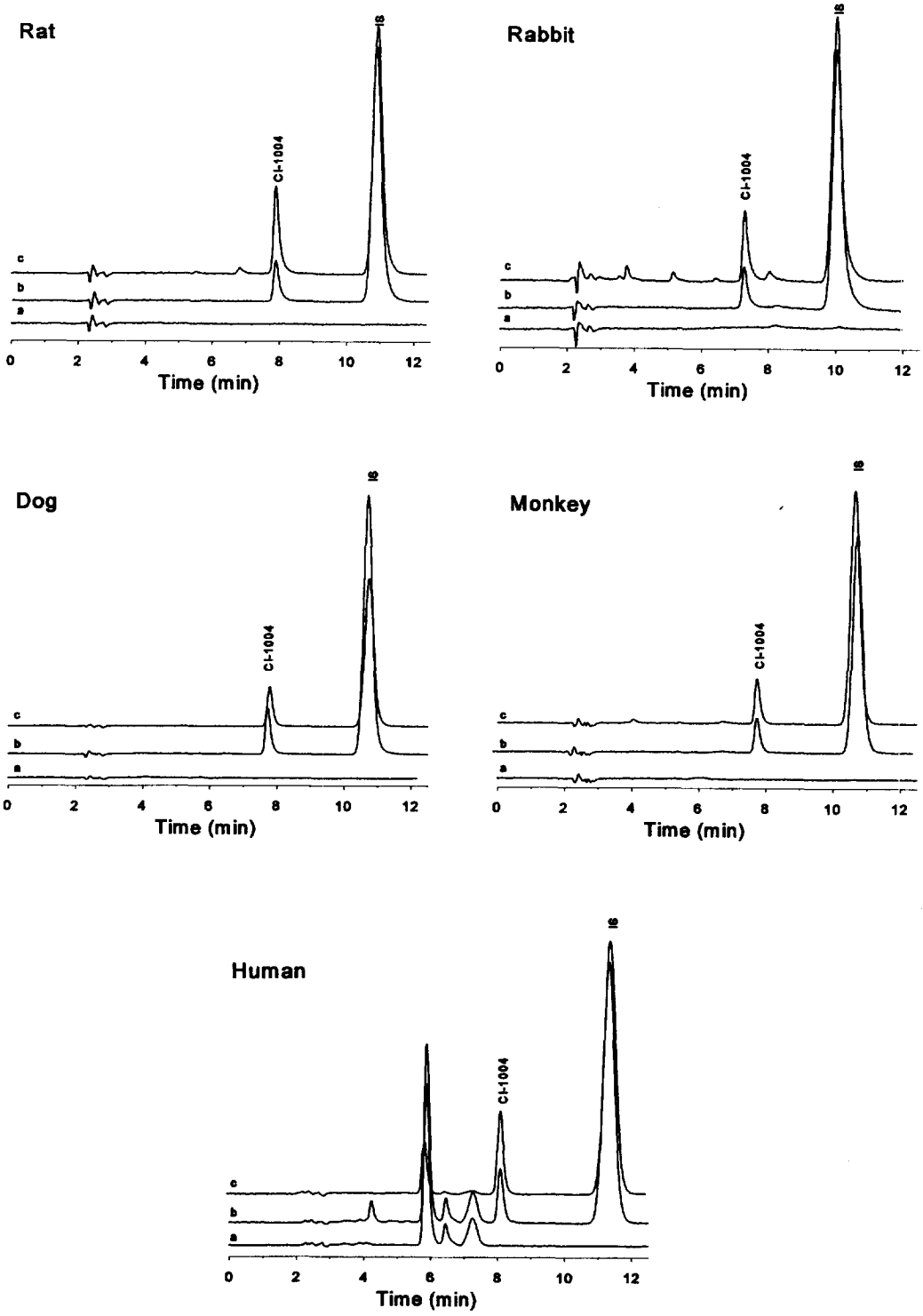

Fig. 2. Chromatograms of extracted blank plasma (a), a 100 ng/ml I calibration standard (b), and unknowns following PO dosing of I (c) in each species.

Table 2
Mean percent recovery (±S.D.) of I and I.S. from rat, rabbit, dog, monkey and human plasma

Species	I			I.S.
	Low[a]	Medium[b]	High[c]	1000 or 1250 ng/ml
Rat	105.0 (5.6)	100.0 (1.1)	104.0 (1.4)	81.9 (1.7)
Rabbit	82.8 (15.9)	92.2 (3.9)	92.7 (3.9)	77.5 (3.1)
Dog	105.0 (1.9)	105.0 (0.98)	107.0 (1.0)	82.0 (2.0)
Monkey	88.1 (14.6)	105.0 (4.7)	95.1 (3.3)	80.6 (1.5)
Human	102.0 (4.1)	94.5 (4.2)	93.1 (5.1)	111.0 (7.1)

[a] 30 ng/ml: rat, rabbit and dog; 10 ng/ml: monkey; 8 ng/ml: human.
[b] 300 ng/ml: rat, rabbit and dog; 100 ng/ml: monkey; 200 ng/ml: human.
[c] 2400 ng/ml: rat and dog; 3000 ng/ml: rabbit; 1000 ng/ml: monkey; 2000 ng/ml: human.

4. Discussion

An earlier, more basic assay was developed for I which used solid-phase extraction and HPLC with a C18 column at ambient temperature. This basic method was used to measure I and numerous analogs in samples obtained from efficacy screening tests during early drug discovery. In these tests, larger doses were used and assay sensitivity was not typically an issue. As I entered lead compound development a more sensitive assay was required. Since I is a basic compound, peak tailing was a problem with the non-base deactivated C18 column employed to that point. Therefore, a switch was made to the Zorbax Rx-C8 column. This switch provided sharper peaks and an immediate improvement in assay sensitivity. Raising the column temperature to 45°C also improved peak shape and shortened the run time of each injection. In addition, modifications to the solid phase extraction method were made that optimized recovery of I, provided a cleaner sample extract and allowed for direct injection onto the HPLC system. As the above results demonstrate, the final method is sensitive, selective, precise and accurate.

The human assay required further method modifications due to endogenous components in human plasma extracts that eluted at or near the retention

Table 3
Intra- and inter-run accuracy and precision for I in rat, rabbit, dog, monkey and human quality controls (n=9 at each level)

Species	Mean concentration of (ng/ml)		Intra-run[a]		Inter-run	
	Nominal	Observed	C.V. (%)	R.E. (%)	C.V. (%)	R.E. (%)
Rat	30	29.0	7.7	−4.3	4.2	−3.3
	300	289	3.6	−4.7	2.4	−3.7
	2400	2290	15.3	−6.7	8.3	−4.6
Rabbit	15	15.1	3.7	4.0	3.5	0.67
	300	303	1.8	2.3	2.0	1.0
	2400	2440	2.2	3.3	1.8	1.7
Dog	6	5.72	7.5	−8.7	5.9	−4.7
	120	117	4.7	−5.0	3.9	−2.5
	2400	2350	4.2	−4.6	3.9	−2.1
Monkey	15	14.8	5.0	−6.0	4.8	−1.3
	300	301	3.5	3.7	3.3	0.33
	2400	2410	2.5	3.3	3.1	0.42
Human	7.81	7.78	10.3	−3.3	6.3	−0.38
	125	128	1.10	5.6	2.9	2.4
	2000	2070	2.40	9.5	4.3	3.5

[a] Values reported reflect the largest intra-day accuracy and precision observed from the three batch runs.

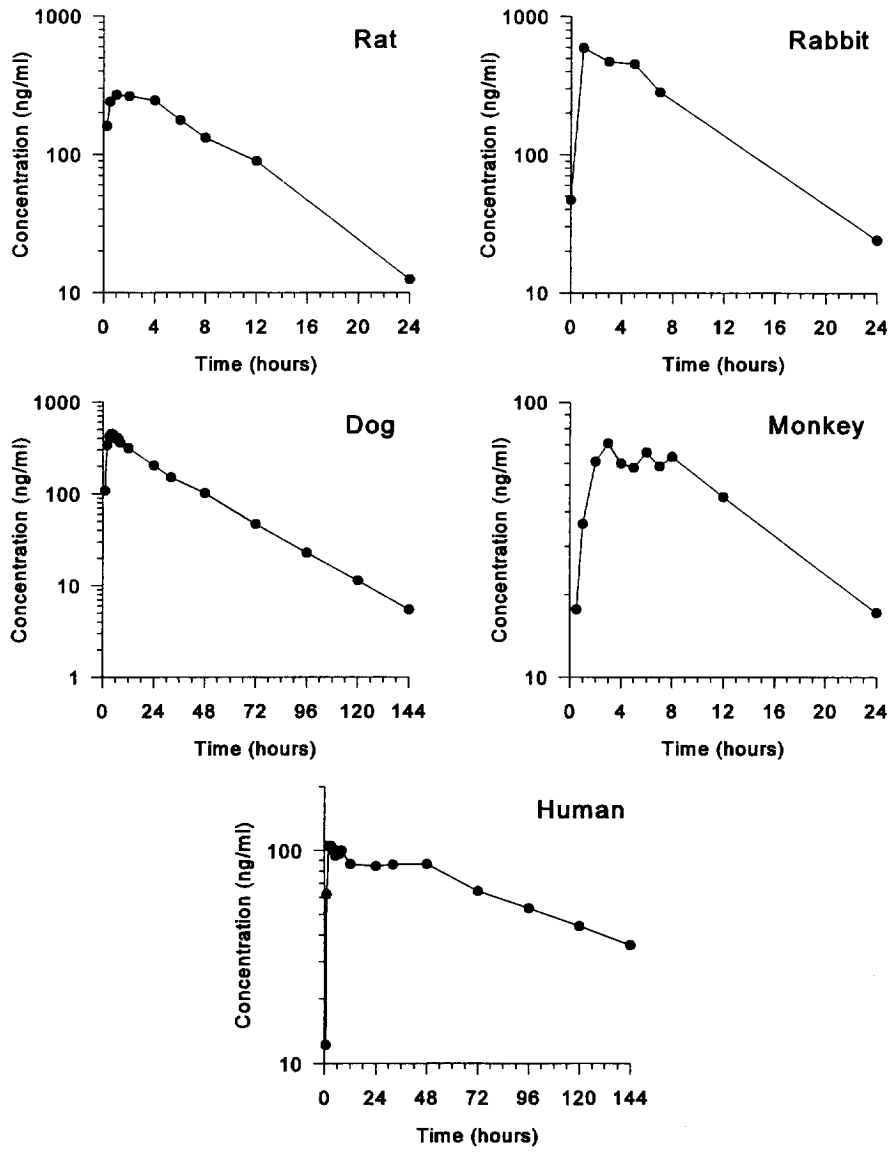

Fig. 3. Representative concentration–time profiles following PO administration of I to each species.

time of I. The interfering peak could not be eliminated with changes made to the solid-phase extraction method such as addition of acid and base preconditioning or washing steps, or modifications of wash solvents. Chromatographic modifications, including replacing all or part of the acetonitrile with methanol, replacing the column with a Zorbax Rx-C18, Zorbax SB-phenyl and Alltech Hypersil BDS CN column were also attempted. However, the desired separation of I from the endogenous peak was not achieved. On the other hand, lowering the column temperature from 45°C to 40°C adequately resolved I from the endogenous substance. Further reduction of temperature to 35°C provided even greater separation but an attendant peak broadening precluded detection of the lowest standard. Another problem encountered during the human assay method development was an increase in variability of I and

I.S. peak heights. This was traced to variability in elution (i.e. recovery) of both I and I.S. from the solid-phase extraction cartridges. This problem was overcome by decreasing the volume of 0.5% NH_4OH added to plasma from 0.5 ml to 0.25 ml and increasing the elution solvent volume from 0.5 ml to 0.75 ml. Dividing the elution solvent volume addition into three 0.25-ml aliquots, with column bed drying between each addition, provided additional consistency in recovery of both compounds.

5. Conclusions

A liquid chromatographic method to quantitate I has been developed and validated in rat, rabbit, dog, monkey and human plasma. The assays are selective, precise, accurate and linear over the concentration ranges studied. These methods were successfully used in preclinical and clinical pharmacokinetic studies of I.

References

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