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Determination of a novel indolylpiperazine anti-migraine agent in rat, monkey, mouse and rabbit plasma by high-performance liquid chromatography with electrochemical detection

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Abstract

A specific, accurate, precise and reproducible assay for the quantitation of a novel indolylpiperazine anti-migraine agent (I) in plasma from various animal species is described. The method involves addition of internal standard (I.S.) and 1.0 *M* sodium carbonate to the plasma sample, vortex-mixing and extraction with ethylene dichloride. The organic layer is then back-extracted in a buffer consisting of 0.1 *M* tetramethylammonium hydroxide (TMAH), pH 3.0 and 0.1 *M* $(NH_4)_2HPO_4$, pH 3.0, in water. The aqueous layer is injected on to a Zorbax cyano analytical column with a mobile phase consisting of acetonitrile, methanol and water (15:5:80, v/v/v) with 0.01 *M* TMAH, pH 3.0 and 0.01 *M* $(NH_4)_2HPO_4$, pH 3.0. The eluate is monitored by electrochemical detection at 0.9 V (guard cell), 0.5 V (detector 1) and 0.8 V (detector 2). The retention times of I and I.S. were 7 and 10 min, respectively. In drug-free control plasma, there were no interfering peaks seen at the retention times of I or I.S. The standard curve was linear over the concentration range of 5–500 ng/ml in rat, monkey, mouse and rabbit plasma. The lower limit of quantitation in all four matrices was 5.0 ng/ml. Within- and between-assay variability of quality control samples was less than 9% relative standard deviation and the predicted concentration of the quality control samples deviated by less than 15% from the nominal concentration. The stability of I was established for up to 36 h in the autosampler tray, up to 10 months in plasma at −20°C and up to 2 h in plasma at room temperature. The assay is validated for determination of I in plasma.

Keywords: Indolylpiperazine

1. Introduction

A novel indolylpiperazine (BMS-180048, I), 3-[3-[4-(5-methoxy-4-pyrimidinyl)-1-piperazinyl]propyl]-N-methyl-1H-indole-5-methanesulfonamide fumarate, is a new chemical entity being developed for its abortive anti-migraine properties (Fig. 1). In pharmacological evaluation with isolated bovine, feline and human middle cerebral artery, canine saphenous vein and guinea pig iliac artery, I has shown 5-HT_1-like agonist activity [1]. Additionally I was devoid of activity in the isolated porcine coronary artery and rat aorta, indicating a lack of effect at peripheral 5-HT_2 receptors [2].

As a crucial part of the drug development process, a rapid, sensitive and specific assay is required to

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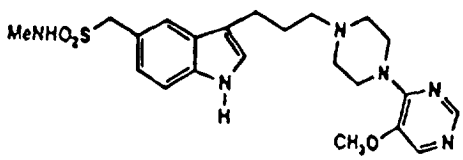
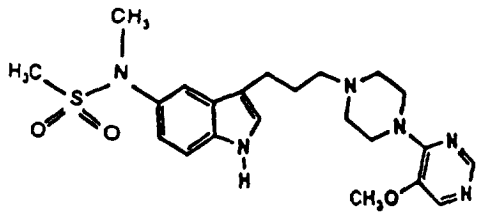


BMS-180048



BMY-46317 (Internal Standard)

Fig. 1. Chemical structures of I and of the internal standard.

measure the drug in plasma in non-clinical and clinical pharmacokinetic studies. In order to analyze plasma samples from rodent toxicokinetic studies, where the sample volume is limited, an assay with electrochemical detection was developed and validated in different species. Although many xenobiotics can readily be detected with fluorescence or UV detection, liquid chromatography combined with electrochemical detection provides a more sensitive alternative [3]. An HPLC method with electrochemical detection has also been successfully employed for the determination of sumatriptan succinate in plasma and urine [4]. The assay validation was focused primarily on rat and monkey, which are the primary species used in the toxicologic evaluation of I. The assay was also extended to mouse for support of carcinogenicity studies and to rabbit for support of reproductive toxicity studies. The assay has also been used to determine concentrations of I in human plasma with slight modification.

2. Experimental

2.1. Chemicals and reagents

Compound I (97.3% purity) and I.S. (99% purity) were synthesized in-house (Fig. 1). HPLC-grade methanol and acetonitrile were obtained from Mallinckrodt Specialty Chemicals (Paris, KY, USA). Control mouse plasma (ethylenediaminetetraacetic acid, EDTA) and control rabbit plasma (EDTA) were obtained from Cocalicio Biologicals (Reamsdale, PA, USA) and control rat plasma (EDTA) and control monkey plasma (EDTA) were obtained from Buckshire (Perkasie, PA, USA) and Cocalico Biologicals (Reamstown, PA, USA), respectively. Ethylene dichloride (HPLC grade) was purchased from Baxter (Muskegon, MI, USA). All other chemicals and reagents were of analytical reagent grade.

2.2. Preparation of solutions of I and of internal standard

Stock solutions of I and I.S. were prepared in absolute ethanol at a concentration of 100 μg/ml, as the free base form and were found to be stable at 5–7°C for up to 3 months. Plasma standards in rat, monkey, mouse and rabbit plasma were prepared on the day of sample analysis by transferring 25 μl of the ethanolic stock solution of I into 4.975 ml of control plasma to yield a 500 ng/ml spiked plasma solution. This solution was subsequently diluted with an appropriate volume of control plasma to prepare a series of standards ranging from 5 to 500 ng/ml. A working solution of the I.S. (500 ng/ml) was prepared on the day of analysis by diluting the stock solution with absolute ethanol.

2.3. Preparation of the extraction buffer

The extraction buffer was prepared by mixing thoroughly 10 ml of 1.0 *M* tetramethylammonium hydroxide (TMAH), pH 3.0, 10 ml of 0.1 *M* ammonium phosphate, dibasic, pH 3.0, and 80 ml of milli Q water. The extraction buffer was stored at room temperature and used for up to 3 months.

2.4. Sample extraction

To the plasma sample (0.5 ml of monkey plasma or 0.2 ml of rat, mouse or rabbit plasma), 50 μl of 500 ng/ml I.S. solution was added, followed by the addition of 0.5 ml of 1.0 *M* sodium carbonate solution. The samples were vortexed on a multi-tube vortexer for 10 s. To each sample tube, 4.0 ml of ethylene dichloride was added and the capped tubes

were rotated on a Roto Rak (Fisher Scientific, Fairlawn, NJ, USA) for 10 min. The samples were then centrifuged at 500 *g* for 5 min. The top, aqueous layer, was aspirated and the bottom, organic layer, was transferred to a clean polypropylene tube containing 0.25 ml of extraction buffer. The tubes were capped and mixed for 10 min on the Roto Rak and then centrifuged at 500 *g* for 5 min. The upper, aqueous, layer was transferred to limited volume inserts and a 75-μl aliquot was injected onto the HPLC system.

2.5. Apparatus and chromatographic conditions

Analyses were performed using a Waters Model 600E system controller, a Waters intelligent sample processor (WISP), a Model 715 Ultra (Waters Associates, Milford, MA, USA) and an ESA Coulochem II detector (ESA, Bedford, MA, USA). The ESA cell employed was the Model 5010 standard analytical cell. The column used was a Zorbax cyano column (250 × 4.6 mm I.D., 5 μm) from Mac Mod (Chadds Ford, PA, USA) maintained at ambient temperature. A Model 3357 Laboratory Automation System from Hewlett Packard (Palo Alto, CA, USA) was used for data acquisition and processing. The electrochemical detector was operated at a guard cell voltage of 0.9 V, a detector 1 voltage of 0.5 V and a detector 2 voltage of 0.8 V at 1 microampere gain which yielded the optimum signal to noise ratio. All voltages used were positive indicating oxidative mode of detection. No offset was used on the detector. The mobile phase consisted of 15% acetonitrile, 5% methanol, 1% of 1.0 *M* TMAH, pH 3.0, and 1% of 1.0 *M* ammonium phosphate, dibasic, pH 3.0, in water and was prepared fresh weekly. The flow-rate was 1.0 ml/min.

2.6. Assay validation

For assay validation, the guidelines proposed at the conference on Analytical Methods Validation for bioavailability, bioequivalence and pharmacokinetic studies were followed [5].

2.6.1. Specificity

Specificity of the assay was evaluated by examination of chromatograms from blank rat, monkey, mouse and rabbit plasma samples for possible endogenous interference with the peaks corresponding to I and I.S.

2.6.2. Range of reliable response

The standard curve parameters from several standard curves were used to establish the range of reliable response in rat, monkey, mouse and rabbit plasma. Eight non-zero standard concentrations were prepared, in duplicate, ranging from 5–500 ng/ml and processed as described (Section 2.4). A weighted linear regression of I to I.S. peak height ratio vs. the concentration of I was carried out using the reciprocal of each concentration as the weighting factor.

2.6.3. Determination of the lower limit of quantitation

For determination of the lower limit of quantitation (LLQ), a sample of the specific matrix was obtained from multiple sources and spiked in ten replicates to contain the concentration to be established as the LLQ of I. For example, to establish the LLQ in rat plasma, each of ten replicates were processed at 2.5 ng/ml and 5.0 ng/ml in rat plasma. A standard curve was prepared, processed and injected along with the samples. Percent deviation of the predicted concentration in each sample was calculated from the nominal concentration. LLQ was defined as the concentration at which at least 70% of the samples assayed were within 20% deviation from the nominal concentration and it represented the lowest standard in the analytical run. LLQ determination was carried out in rat, monkey, mouse and rabbit plasma.

2.6.4. Accuracy and precision

Intra- and inter-assay accuracy and precision were determined by analyzing quality control (QC) samples prepared in rat and monkey plasma at concentrations which fell within the lower, within the second and third, and within the upper quartile of the standard curve range. A fourth concentration was spiked above the standard curve range and diluted appropriately before extraction to demonstrate the accuracy and precision of analysis where dilution is required. Five replicate QC samples at each concentration were analyzed together with eight standards (in duplicate) in a single analytical run on three different days. The intra- and inter-assay (within-day

and between-day) precision was determined by one-way analysis of variance.

2.6.5. Stability

Stability of I in the injection solvent obtained following extraction of spiked rat and monkey plasma, as well as those obtained from animals treated with I, was assessed over a 36 h injection period. Stability was assessed by comparing the absolute peak height of I and the peak height ratio of I to the I.S. at time "*t*" to peak height and to the peak height ratio of the first injection (time "zero"). Stability of the internal standard was also assessed by comparing the absolute peak heights over 36 h. Freeze–thaw stability of I in plasma was assessed by analyzing freshly prepared QC samples and comparing the predicted concentration after three consecutive freeze–thaw cycles. Stability of I in plasma during sample processing at room temperature was also assessed. A set of spiked samples of rat and monkey plasma, as well as samples obtained from treated animals, were thawed and processed after the samples were allowed to sit at room temperature for at least 2 h, and the predicted concentrations were compared with samples processed without any time delay after thawing. Long-term stability of I in spiked rat and monkey plasma was assessed over a period of 10 months under storage conditions of −20°C. Long-term stability of I, at −20°C, was also assessed in plasma samples obtained from drug-treated animals over a period of 2 months. Plasma samples obtained from drug-treated animals (in vivo samples) were included in the assessment of stability wherever possible, since it is generally speculated that one or more metabolites of the drug may influence the stability profile of the drug being analyzed.

2.7. Application of the assay to pharmacokinetic evaluation in rats

In a one-month oral toxicity study, I was administered at 12, 70 and 400 mg/kg body weight, by gavage once daily. Satellite groups consisting of nine male and nine female rats, at each dose level, were included for the determination of I in plasma. Blood samples were collected at approximately 0.5, 1, 2, 4, 8 and 24 h post-dose on day 1 and day 24 of the study. Plasma was separated by centrifugation (1000 *g*) and stored frozen at −20°C until analysis. Plasma samples were extracted and analyzed as described earlier, along with standards and QC samples in each analytical run (Section 2.4. The highest observed plasma concentration (C_{max}) and the area under the plasma concentration curve from 0 to the last quantifiable time point (AUC (0–T)) were calculated for assessment of the pharmacokinetics of I.

3. Results and discussion

3.1. Specificity

No interfering peaks were found at the retention times of I or of the I.S. in extracted rat, monkey, mouse and rabbit plasma, that could significantly affect the quantitation of I. Sample chromatograms of blank and spiked monkey plasma are shown in Fig. 2.

3.2. Range of reliable response

The assay was found to be linear over the range of 5–500 ng/ml of I in rat and monkey plasma. The summary of standard curve parameters is presented in Table 1. The assay was also found to be linear over the range of 5–500 ng/ml in mouse and rabbit plasma.

3.3. Lower limit of quantitation

The results for determination of the lower limit of quantitation are summarized in Table 2 for rat, monkey, mouse and rabbit plasma. In monkey plasma, five of the ten samples spiked at 2.5 ng/ml were outside the acceptable ± 20% deviation limits, whereas, at 5.0 ng/ml, seven of the ten samples were within 13% of the nominal concentration. In rat plasma, six of the ten samples were outside acceptable limits, at 2.5 ng/ml, while eight of the ten samples spiked at 5.0 ng/ml were within 15.6% of the nominal concentration. In mouse plasma, eight of the ten samples spiked at 5.0 ng/ml were within

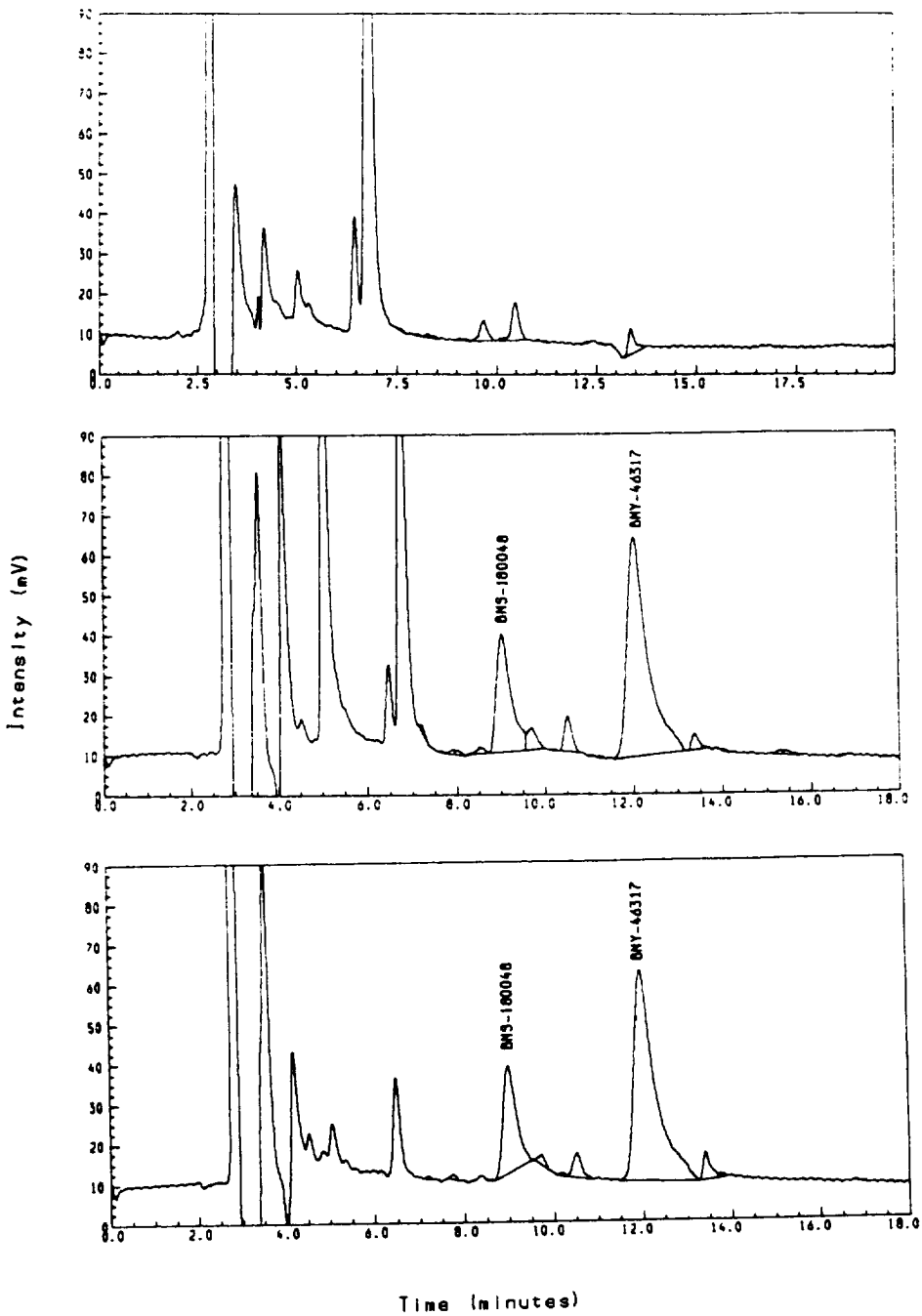


Fig. 2. Chromatograms for I and I.S. in monkey plasma. Top = blank, middle = spiked standard and bottom = study sample.

17.4% of the nominal concentration, while at 10.0 ng/ml, all samples were within 5.7% of the nominal concentration. In rabbit plasma, all the samples spiked at 5.0 and 10.0 ng/ml were within acceptable limits. Based on these results, the LLQ was established at 5.0 ng/ml, in all the species evaluated.

Table 1
Standard curve summary of rat and monkey plasma

Run No.	Slope	Intercept	r^2
Rat plasma			
1	0.01269	−0.03219	0.996
2	0.01359	−0.02610	0.996
3	0.01255	−0.02700	0.996
n	3	3	
Mean	0.01295	−0.02843	
S.D.	0.00057	0.00329	
Monkey plasma			
1	0.03186	−0.04751	0.998
2	0.03244	−0.08338	0.997
3	0.03305	−0.06859	0.998
n	3	3	
Mean	0.03245	−0.06649	
S.D.	0.00059	0.01803	

3.4. Accuracy and precision

The intra- and inter-assay variability expressed as the percentage relative standard deviation [R.S.D. (%)] was less than 6% in rat plasma and less than 9% in monkey plasma, at the four concentrations analyzed (Table 3). Mean predicted concentration was within 7% of the nominal concentration in rat plasma and within 14% of the nominal concentration in monkey plasma. The accuracy and precision of the assay was thus found to be acceptable for the analysis of plasma samples, in support of pharmacokinetic studies.

3.5. Stability

The results of studies on the stability of I in the injection solvent, following extraction of rat and

Table 2
Determination of the lower limit of quantitation (LLQ) in rat, monkey, mouse and rabbit plasma

Replicate No.	Rat plasma		Monkey plasma		Mouse plasma		Rabbit plasma	
	Predicted concentration (ng/ml)	Deviation (%)	Predicted concentration (ng/ml)	Deviation (%)	Predicted concentration (ng/ml)	Deviation (%)	Predicted concentration (ng/ml)	Deviation (%)
	2.5 ng/ml[a]		2.5 ng/ml[a]		5.0 ng/ml[a]		5.0 ng/ml[a]	
1	3.40	36.1	4.48	79.0	5.87	17.4	4.97	−0.6
2	2.77	10.7	3.00	19.8	5.57	11.3	5.14	2.7
3	4.04	61.5	3.26	30.2	4.81	−3.7	5.26	5.1
4	4.55	82.1	2.83	13.2	5.78	15.5	5.68	13.6
5	4.47	78.9	2.89	15.4	6.36	27.2	5.21	4.1
6	2.91	16.2	2.97	18.9	4.28	−14.3	5.22	4.4
7	3.43	36.7	3.04	21.6	3.99	−20.2	4.69	−6.1
8	2.87	14.9	8.92	256.6	5.43	8.6	5.28	5.6
9	3.79	49.9	2.90	16.0	5.63	12.6	5.33	6.7
10	3.01	20.3	3.48	39.3	4.95	−1.1	5.03	0.6
	5.0 ng/ml[a]		5.0 ng/ml[a]		10.0 ng/ml[a]		10.0 ng/ml[a]	
1	4.84	−3.2	10.87	117	9.78	−2.2	10.94	9.4
2	4.90	−2.0	14.59	192	10.21	2.1	10.92	9.2
3	7.51	50.2	4.54	−9.2	10.13	1.3	10.99	9.9
4	6.69	33.8	5.45	8.9	10.08	0.8	9.03	−9.7
5	5.87	15.6	5.64	12.9	9.73	−2.7	11.15	11.5
6	5.03	0.5	4.79	−4.2	9.81	−1.9	10.91	9.1
7	4.38	−12.3	8.22	64.3	10.20	2.0	11.09	10.9
8	5.01	0.1	4.70	−6.1	10.15	1.5	11.33	13.3
9	5.39	7.9	4.70	−6.0	9.43	−5.7	11.00	10.0
10	5.57	11.3	5.46	9.3	10.02	0.2	10.56	5.6

[a] Nominal concentration.

Table 3
Inter-assay and intra-assay accuracy and precision in rat and monkey plasma

Nominal concentration (ng/ml)	n[a]	Mean observed concentration (ng/ml)	Deviation (%)	Precision (R.S.D., %)	
				Between-run	Within-run
Rat plasma					
17.0	13	17.95	5.59	2.59	2.69
126	13	124.51	−1.18	4.00	4.58
360	15	365.43	1.51	3.57	4.30
1800	14	1922.08	6.78	2.93	5.80
Monkey plasma					
18.00	15	17.11	−4.94	4.00	8.22
120	15	103.90	−13.42	3.40	2.82
375	15	373.51	−0.40	3.15	2.84
1800	15	1712.58	−4.86	4.87	6.31

[a] Five replicates of each concentration were analyzed in three separate analytical runs on three different days. n is not always 15, since some samples were rejected due to chromatographic anomalies.

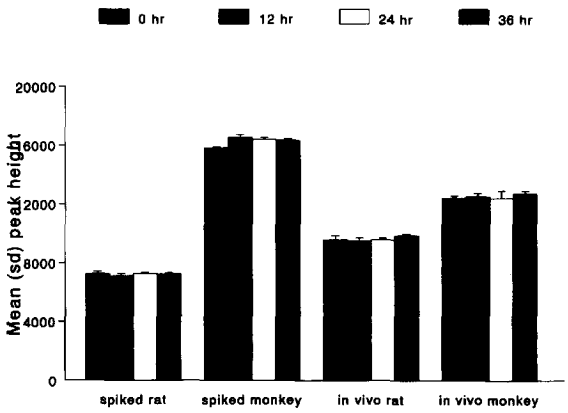


Fig. 3. Autosampler stability of I over 36 h. Samples used were spiked rat plasma, spiked monkey plasma, in vivo rat plasma and in vivo monkey plasma.

monkey plasma samples over 36 h are shown in Fig. 3. Mean peak height at time "t" relative to the mean peak height at time "zero" ranged from 99.1 to 104% over 36 h. Similar results were obtained when the peak height ratios of I to the I.S. were compared over 36 h. I was thus found to be stable in the extraction buffer at room temperature in samples extracted from spiked rat and monkey plasma and from the plasma of drug-treated animals.

Freeze–thaw stability of I in plasma was assessed over three freeze–thaw cycles at two concentrations (Table 4). The predicted concentrations deviated by less than 8% from the corresponding nominal concentrations, indicating that the stability of I over three freeze–thaw cycles was acceptable. Stability data for I, in rat and monkey plasma at room

Table 4
Freeze–thaw stability

Nominal concentration (ng/ml)	Freeze–thaw cycle	Predicted concentration (ng/ml)	Precision (R.S.D., %)	Accuracy (% deviation from nominal)
40.0	0	40.39	6.7	1.0
	1	37.95	3.9	−5.1
	2	37.07	7.1	−7.3
	3	42.30	1.1	5.7
800	0	822.55	0.5	2.8
	1	787.73	0.7	−1.5
	2	809.11	6.1	1.1
	3	793.90	0.8	−0.8

Table 5
Stability of rat and monkey plasma at room temperature, during sample extraction

Sample identification	Predicted concentration (ng/ml)		Deviation from 0 h (%)
	0 h[a]	2 h[a]	
Rat QC	1815.65	1947.89	7.28
Monkey QC	155.49	150.94	−2.93
Rat in vivo sample	3991.60	3799.51	−4.81
Monkey in vivo sample	8105.42	8853.96	9.24

[a] Time elapsed at room temperature.

temperature, is summarized in Table 5. QC samples and in vivo samples left at room temperature for 2 h before extraction, had predicted concentrations within 10% of those extracted without any time delay at room temperature. Thus, there appeared to be no evidence of analyte degradation in rat and monkey plasma over a 2 h time period, at room temperature.

QC samples prepared in rat plasma were stable for at least 9 months and those in monkey plasma were stable for at least 10 months, at −20°C (Table 6). I was found to be stable in plasma samples obtained from treated animals for at least 2 months. The range of concentrations evaluated extended from 14 ng/ml to 7694 ng/ml. The predicted concentration ranged from −12% to +16% of the nominal concentration at various times of storage. Overall, I was found to be stable in rat and monkey plasma at, −20°C, over a prolonged period of storage.

3.6. Pharmacokinetics in rats

Mean plasma concentration–time profile of I, on day 24 in a one-month toxicity study in rats, is shown in Fig. 4. Female rats showed consistently higher plasma concentrations of I than did male rats. On day 24, the C_{max} values were 45.5, 2085 and 15 905 ng/ml in male rats and 784, 4282 and 22 537

Table 6
Long-term stability in rat and monkey plasma

Sample identification	Nominal concentration (ng/ml)	Duration of storage at −20°C (months)	Predicted concentration (ng/ml)	Deviation from nominal concentration (%)
Rat QC	15	3	14.91	−0.6
Rat QC	370		399.19	7.9
Rat QC	13	5	14.20	9.2
Rat QC	362		389.72	7.7
Rat QC	125	6	134.92	7.9
Rat QC	360		348.36	−3.2
Rat QC	40	8	36.92	−7.7
Rat QC	40	9	38.57	−5.8
Rat QC	800		789.38	−1.3
Rat study sample		2	655.72[a]	−8.6[c]
			599.20[b]	
Monkey QC	40	2	45.56	13.9
Monkey QC	800		925.41	15.7
Monkey QC	30	6	27.97	−6.8
Monkey QC	110		97.05	−11.8
Monkey QC	51	7	53.66	5.2
Monkey QC	1400		1273.54	−9.0
Monkey QC	40	9	38.57	−3.6
Monkey QC	5000		4949.85	−1.6
Monkey QC	40	10	42.26	5.7
Monkey study sample		2	6758.27[a]	13.8[c]
			7693.72[b]	

[a] Predicted concentration in the first run.
[b] Predicted concentration 2 months after the first run.
[c] Percentage deviation of the second value from the first.
Samples above ULQ were diluted appropriately prior to analysis.

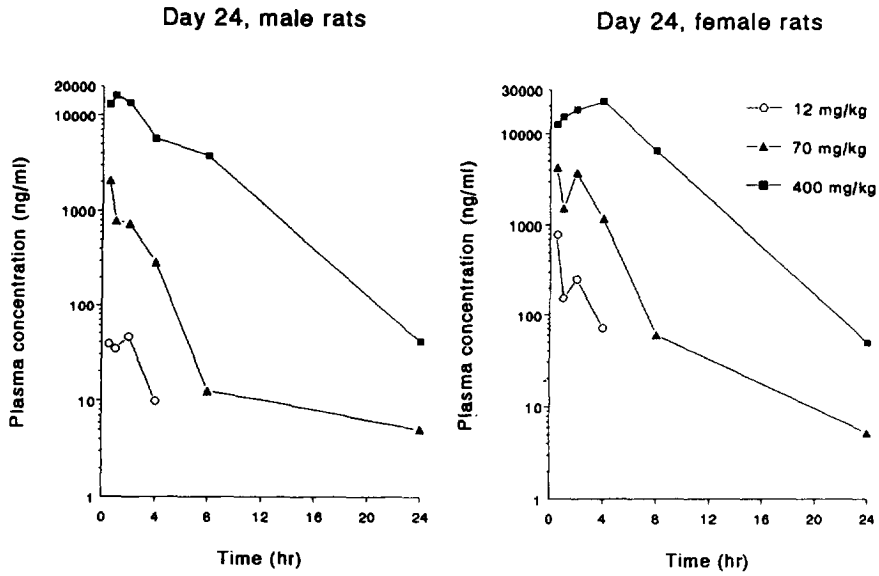


Fig. 4. Plasma concentration–time profile of I on day 24 in a one-month rat toxicology study of male and female rats. Doses used were 12, 70 and 400 mg/kg body weight, administered by oral gavage once daily.

ng/ml in female rats at doses of 12, 70 and 400 mg/kg body weight, respectively. AUC (0–T) values on day 24 were 122, 3777 and 94 099 ng.h/ml in male rats and 952, 12 998 and 178 686 ng.h/ml in female rats, at the three doses. Exposure to I, as indicated by C_{max} and AUC (0–T) increased out of proportion to the dose administered, over the dose range of 12 to 400 mg/kg body weight.

The HPLC procedure with electrochemical detection (HPLC–EC) has been extensively used in various non-clinical pharmacokinetic and toxicokinetic studies of I. The 5–10 fold increased sensitivity of the electrochemical detection was an advantage over a previously developed HPLC procedure used in conjunction with UV detection. The present HPLC–EC method, with slight modification, has also been used extensively in the analysis of human plasma samples from Phase I clinical studies. In summary, the HPLC–EC assay method for the determination of I in plasma, is accurate, precise and reproducible. It is specific and sufficiently sensitive to elucidate the pharmacokinetics of I.

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