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Analysis of a novel antiinflammatory agent, 1-(7-*tert.*-butyl-2,3-dihydro-3,3-dimethylbenzo[*b*]furan-5-yl)-4-cyclopropylbutan-1-one (PGV-20229), in plasma matrices by stable-isotope-dilution gas chromatography–mass spectrometry

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Abstract

A sensitive and selective GC–MS method was developed for the determination of low levels of a novel antiinflammatory agent, 1-(7-*tert.*-butyl-2,3-dihydro-3,3-dimethylbenzo[*b*]furan-5-yl)-4-cyclopropylbutan-1-one (I), in small volumes of animal plasma. The method involved the addition of $^{13}C_6$-labeled-I to plasma samples, followed by a simple liquid–liquid extraction with hexane to isolate the analytes from matrix components. The levels of I in the sample extracts were determined by isotope-dilution GC–MS analysis using selected-ion monitoring. The method was linear over three orders of magnitude, with a limit of quantitation of 1.8 ng/ml I, using plasma sample volumes of 0.1 ml. The method was utilized to determine the pharmacokinetic parameters of I in rats and dogs, following intravenous administration. © 1997 Elsevier Science B.V.

Keywords: Stable-isotope dilution; PGV-20229; 1-(7-*tert.*-Butyl-2,3-dihydro-3,3-dimethylbenzo[*b*]furan-5-yl)-4-cyclopropylbutan-1-one

1. Introduction

Rheumatoid arthritis (RA), a disease of unknown etiology, is characterized by chronic inflammation, pain and destruction of the articular joint [1]. First-line therapy involves the use of non-steroidal antiinflammatory drugs (NSAID) to treat the pain and inflammation associated with the disease. All currently marketed NSAIDs share a common mechanism of action, i.e. their ability to inhibit the cyclooxygenase (COX) pathway of arachidonic acid metabolism that leads to the production of proinflammatory prostaglandins [2]. This common mechanism of action is hypothesized to be the cause of a major NSAID-induced side effect, gastrointestinal irritation and ulceration, since prostaglandins are also cytoprotective in the stomach [3,4]. Recently, two isoforms of the COX enzyme have been identified. COX-1 is constitutively expressed and is thought to be responsible for maintaining homeostatic levels of

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prostaglandins, while the second isoform, COX-2, is inducible and is thought to play the major role in acute and chronic inflammation [5,6]. The development of compounds that selectively inhibit COX-2 may result in RA therapies free of the gastrointestinal side effects associated with traditional NSAIDs [7].

Compound I is a novel dihydrobenzofuran derivative, structurally similar to the di-*tert.*-butylphenol class of NSAIDs. It has been shown to possess a greater than 50-fold selectivity for the COX-2 isoform, as well as being an inhibitor of the lipoxygenase pathway of arachidonic acid (Procter & Gamble Pharmaceuticals, unpublished data). Additionally, I has been demonstrated, in animal models, to possess a greatly improved gastrointestinal safety profile relative to traditional NSAIDs (Procter & Gamble Pharmaceuticals, unpublished data). In order to support detailed evaluation of I in various animal models of efficacy, toxicity and pharmacokinetics, analytical methodology was needed to monitor systemic parent drug levels. The rat was used for many of the efficacy, toxicology and pharmacokinetic studies and only limited serial blood volumes could be removed without compromising the animals. Therefore, it was necessary to develop analytical methodology capable of measuring trace levels of I in small volumes (100 μl) of plasma. To meet the analysis needs, a sensitive and selective stable-isotope based gas chromatography–mass spectrometry (GC–MS) method was developed, in conjunction with a simple liquid–liquid extraction (LLE) procedure, for the quantitation of I in small volumes of rat and dog plasma.

2. Experimental

2.1. Chemicals and reagents

Blank rat and dog plasma was obtained from Pel-Freez Biologicals (Rogers, AR, USA). Hexane (HPLC Grade) was from J.T. Baker (Phillipsburg, PA, USA). I (see Fig. 1) and $^{13}C_6$-ring-labeled-I ($^{13}C_6$-I, see Fig. 1) were prepared at Procter & Gamble Pharmaceuticals Health Care Research Center (Mason, OH, USA).

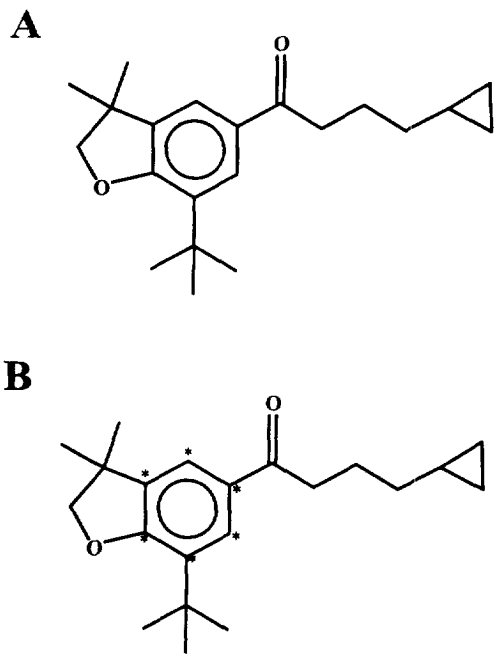

Fig. 1. Structure of: (A) I and (B) $^{13}C_6$-I. The * symbols denote the location of the ^{13}C-labels.

2.2. Preparation of I plasma standards

Separate 10 μg/ml stock solutions of I was prepared in blank rat and dog plasma. Serial dilutions of each stock solution with the appropriate blank plasma were done to provide working matrix standards covering a concentration range from 0.5 to 2000 ng/ml I. A total of 12 standards were prepared, with four standards per decade interval of concentration. Standards were prepared fresh on each day of analysis.

2.3. Preparation of I-spiked plasma samples

I-spiked plasma control samples were prepared in a manner similar to that described above for the standards. Dog and rat I plasma controls were prepared at the 1.8, 18, 178 and 1780 ng/ml level.

2.4. LLE sample preparation procedure

An aliquot, 0.1 ml, of standard, control or unknown plasma samples, dog or rat, were added to

1-ml glass screw-top vials containing 1.6 ng of $^{13}C_6$-I and vortexed. Then 0.20 ml of hexane was added to the vials, the vials were capped with Teflon-lined caps and the contents mixed by vortexing. The vials were placed on a rocker for 10 min, and following rocking the hexane layer was isolated for each sample. The isolated hexane layers were transferred to individual small volume autosampler vials, the volume of hexane was reduced using a stream of nitrogen and the vials were then capped and injected directly.

2.5. Relative recovery of I

The relative recovery of I from the LLE procedure was evaluated for both rat and dog plasma matrices. Aliquots, 0.1 ml, of blank plasma spiked with 1.8 or 178 ng/ml I were extracted with 0.20 ml of hexane, in the absence of internal standard, as described above. The internal standard, 1.6 ng of $^{13}C_6$-I, was then added to the isolated hexane extracts prior to injection. Each concentration level was extracted in replicate, $n=4$ and $n=5$ for the 1.8 and 178 ng/ml I-spiked levels, respectively.

2.6. Stability of I during sample preparation

The stability of I in rat and dog plasma during the sample preparation procedure was examined by incubating the drug in the plasma matrix at ambient temperature. A large volume of rat and dog plasma was spiked to contain 17.8 ng/ml I and then maintained at ambient temperature over a 120-min period. Aliquots, 0.1 ml, of the spiked plasma samples were withdrawn, in triplicate, at 0, 30, 60 and 120 min. The samples were added to tubes containing 1.6 ng of $^{13}C_6$-I, extracted with hexane as described above and analyzed by GC–MS.

2.7. Freeze–thaw stability of I plasma samples

The stability of the drug, in plasma, to repeated freeze–thaw cycles was examined by taking plasma samples spiked with I at the 1.8- and 178-ng/ml levels through three freeze–thaw cycles. Following the third cycle, repetitive ($n=5$) aliquots (0.1 ml) of each sample were added to tubes containing 1.6 ng of $^{13}C_6$-I, extracted with hexane as described above and analyzed by GC–MS.

2.8. Stability of I in LLE extracts

The stability of I in the hexane extracts obtained from the LLE procedure was also examined for both rat and dog plasma samples. Aliquots, 0.1 ml, of standards and plasma samples spiked with 1.8, 18 and 178 ng/ml I were added to tubes containing 1.6 ng of $^{13}C_6$-I and extracted with hexane as described above. Each concentration was prepared in triplicate. The hexane extracts were then analyzed immediately, after sitting 16 h on the autosampler at ambient temperature and after storage for 5 days at 4°C.

2.9. GC–MS conditions

A Hewlett-Packard (HP, Palo Alto, CA, USA) Model 5890A Series II gas chromatograph, an HP Model 5989A mass spectrometer and a Model 7376A autosampler were used with a J&W Scientific (Folsom, CA, USA) DB-5 capillary column (30 m×0.25 mm I.D., 0.1 μm film thickness) for the GC–MS analysis. Helium was used as the carrier gas. The injection port contained a deactivated 4-mm straight liner packed lightly with silinized glass wool. A 3-μl volume was injected in the splitless mode, with the split vent opening at 1.0 min. A pressure program was utilized consisting of an initial pressure of 207 kPa for 1 min, followed by a 669-kPa/min ramp to 69 kPa and a final hold at 69 kPa for 10 min. The thermal program involved an initial isothermal hold at 120°C for 1 min, followed by a linear ramp (15°C/min) to 260°C, a second linear ramp began immediately (70°C/min) to 300°C, and a final hold at 300°C for 2.5 min. The injection port and transfer line were held at 250 and 300°C, respectively. For SIM, a dwell time of 110 ms each was used to monitor m/z 231.2 (I) and m/z 237.2 ($^{13}C_6$-I). Full-scan mass spectra were obtained by scanning a mass range from m/z 50 to 350 using an electron energy of 70 eV.

2.10. Data analysis

The peak area ratio (PAR) for each standard was obtained by dividing the peak area obtained for I by

the peak area obtained for $^{13}C_6$-I. A response factor (RF) was obtained by dividing the mass of I in the standard by the PAR and an average response factor (ARF) was then calculated for all the standards. The concentration of I in control and unknown samples was determined by multiplying the PAR obtained for the sample by the ARF and dividing by the sample volume.

2.11. Rat and dog pharmacokinetic studies

2.11.1. Rats

Male Sprague–Dawley rats, approximately 300–350 g, were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA). They were housed in suspended stainless steel cages and acclimated to standard laboratory conditions of a 12-h

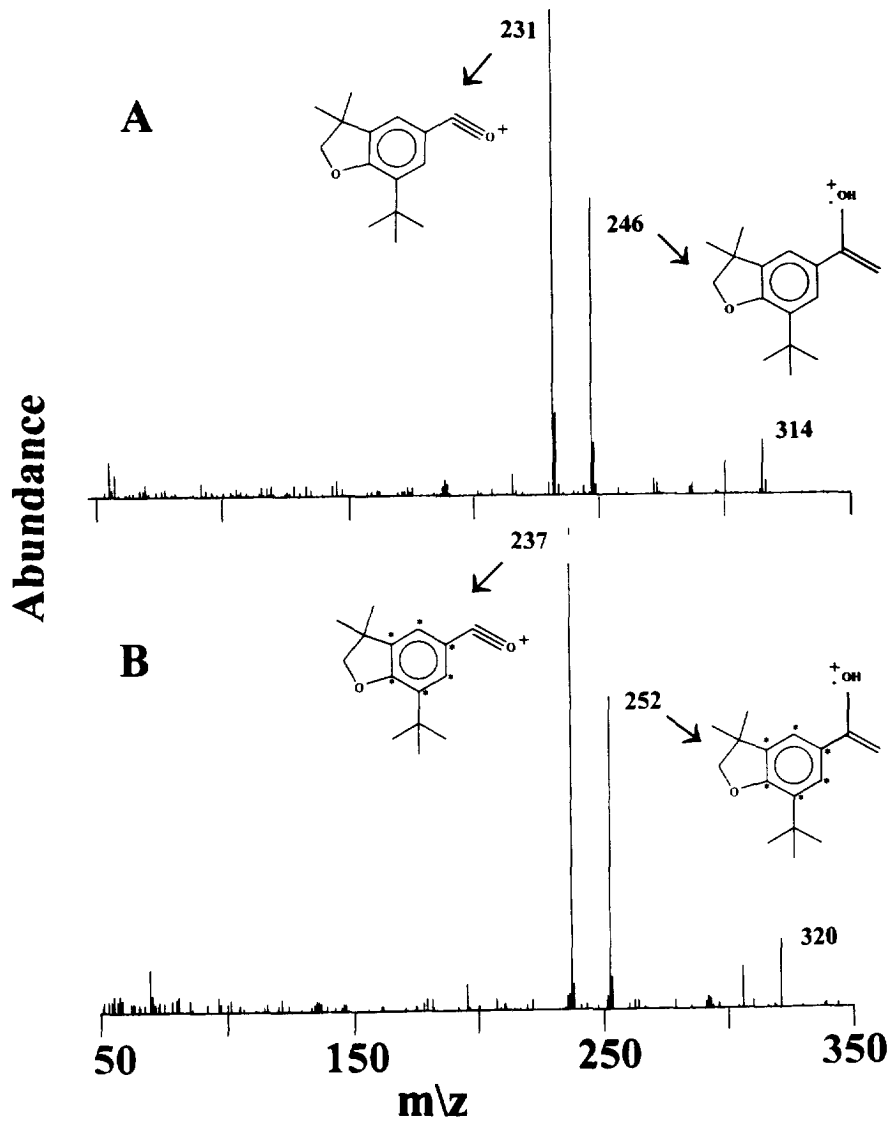

Fig. 2. Electron impact (70 eV) mass spectrum for: (A) I and (B) $^{13}C_6$-I. The * symbols denote the location of the ^{13}C-labels in the fragment ions.

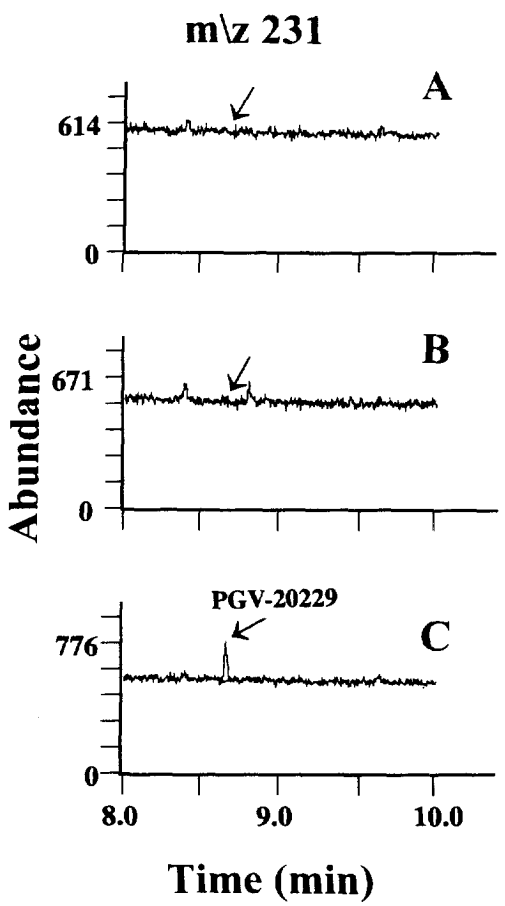

Fig. 3. Selected-ion monitoring profiles for I (*m/z* 231 ion) obtained for: (A) blank rat plasma, (B) blank rat plasma spiked with 16 ng/ml $^{13}C_6$-I, and (C) blank rat plasma spiked with 1.8 ng/ml I and 16 ng/ml $^{13}C_6$-I. The arrows indicates the retention time for I.

light/dark cycle for 3 days prior to dosing. Animals were allowed free access to food (Purina Mills, Richmond, IN, USA) and water throughout the study.

2.11.2. Dogs

Male beagles were obtained from Marshall Farms (North Rose, NY, USA) and maintained in an in-house colony under standard laboratory conditions of a 12-h light/dark cycle. Animals were fed standard laboratory canine chow (Purina Mills). Animals were allowed free access to food and water, except for an overnight fast prior to dosing, and food was withheld for approximately 4 h after dosing.

Intravenous dosing formulations were prepared by dissolving I in a co-solvent system of ethanol–poly-(ethylene glycol)–propylene glycol (10:45:45) to the desired concentration. Prior to administration, solutions were sterilized by filtration through a 0.22-μm membrane filter.

Compound I was administered to rats as an intravenous bolus injection (10 mg/kg) under ether anesthesia into a surgically exposed femoral vein. Following dosing, the incision was closed with wound clips. Blood samples (200–300 μl) were obtained from the tail vein at various times up to 120 h after dosing and collected into EDTA-treated Microtainer tubes. Blood was centrifuged to obtain plasma, and the plasma was stored at −70°C until the time of analysis.

Compound I was administered to dogs as a 10-min intravenous infusion (5 mg/kg) into the cephalic vein, with the aid of a peristaltic infusion pump. Following dosing, the incision was closed with wound clips. Blood samples (3–5 ml) were obtained at various times up to 72 h after dosing, and collected into EDTA-containing Vacutainer tubes. Blood was centrifuged to obtain plasma, and the plasma was stored at −70°C until time of analyses.

2.12. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by non-compartmental techniques using Pharm-NCA© (Simed). Area under the plasma concentration curve (AUC) was calculated using linear trapezoidal rule. Clearance (Cl) was calculated as dose/AUC. apparent volume of distribution (V_z) and volume of distribution steady state (V_{ss}) were estimated according to Eqs. (1) and (2), respectively, where λ_z is the elimination rate constant. Mean residence time (MRT) was calculated as the AUC/AUMC, where AUMC is the area under the first moment curve (concentration×time vs. time).

$$V_z = \frac{Cl}{\lambda_z} \quad (1)$$

$$V_{ss} = MRT \cdot Cl \quad (2)$$

3. Results

3.1. Full scan mass spectra

The mass spectra obtained for I and $^{13}C_6$-I are shown in Fig. 2. A small parent ion was observed for I and $^{13}C_6$-I at *m/z* 314 and 320, respectively. However, the major ion for I and $^{13}C_6$-I occurred at *m/z* 231 and 237, respectively, and these ions were subsequently selected for SIM in the GC–MS quantitative analysis.

3.2. Selectivity, accuracy and precision

Typical SIM chromatographic profiles of the *m/z* 231 ion obtained for blank rat plasma, blank rat plasma spiked with 16 ng/ml $^{13}C_6$-I and blank rat plasma spiked with 1.8 ng/ml I and 16 ng/ml $^{13}C_6$-I are shown in Fig. 3. The combination of the simple LLE sample preparation and SIM monitoring resulted in a chromatographic profile free of endogenous interferences in the retention range of I. The SIM profiles obtained for the *m/z* 237 channel used to monitor the $^{13}C_6$-I were also free of interference (data not shown). Similar results were obtained for dog plasma samples (data not shown). Plots of the PARs for standards were linear over three orders of magnitude, with a correlation coefficients of 0.999 or greater. Accuracy and precision for the analysis of I-spiked plasma samples was determined on three separate days for both rat (Table 1) and dog (Table 2) samples. In general, the accuracy of the I analysis, expressed as the percent recovery, was within 10% of the target value for spiked drug levels from 1.8 to 1780 ng/ml. Precision, as assessed by %R.S.D., over all 3 days was, in general, less than 6% for all I-spiked plasma levels.

Table 1
Accuracy and precision of I analysis in rat plasma

Spiked [I] (ng/ml)	Recovery (%R.S.D.)		
	Day 1	Day 2	Day 3
1.8	104 (4.7%)	108 (3.8%)	126 (3.5%)
18	99 (0.8%)	107 (3.1%)	109 (5.9%)
178	102 (4.2%)	100 (5.1%)	100 (5.2%)
1780	100 (4.5%)	95 (2.7%)	95 (1.9%)

Table 2
Accuracy and precision of I analysis in dog plasma

Spiked [I] (ng/ml)	Recovery (%R.S.D.)		
	Day 1	Day 2	Day 3
1.8	106 (8.5%)	115 (4.5%)	114 (4.8%)
18	105 (1.5%)	110 (0.5%)	108 (2.3%)
178	107 (1.0%)	105 (0.9%)	108 (1.0%)
1780	106 (3.3%)	109 (5.1%)	106 (1.5%)

3.3. Relative recovery and stability of I in LLE extracts

Although the use of a stable-isotope internal standard should correct for any loss of analyte, it is still important to assess the recovery of the drug from the sample preparation step. The relative recovery of I from the LLE procedure was examined by extracting spiked plasma samples in the absence of the stable-isotope internal standard. The internal standard was added to the hexane extracts just prior to injection. The relative recovery of I from rat plasma samples spiked at the 1.8 and 178 ng/ml levels were 65±4 and 53±1%, respectively. Similarly, the relative recovery from dog plasma spiked with I, at the 1.8 and 178 ng/ml levels, were 59±3 and 48±5%, respectively. Clearly, the single hexane extraction left significant amounts of drug in the plasma matrix. Although a second extraction resulted in >85% relative recovery, a single extraction was employed to simplify the method. Additionally, the stable-isotope internal standard corrected for the loss of analyte during the LLE step, as indicated by the accuracy and precision data presented in Tables 1 and 2.

3.4. Plasma and freeze–thaw stability of I

Although the time required to prepare a group of plasma samples by the LLE procedure was less than 1 h, the stability of I in spiked dog and rat plasma

was examined at ambient temperature over a 2-h period. Plasma samples spiked with I (17.8 ng/ml) were withdrawn at various time points over this period, spiked with internal standard, extracted and analyzed. The levels of I found at each time point were within 5% of the initial value obtained at time zero. Additionally, I was also found to be stable, in both rat and dog plasma, through three freeze–thaw cycles. The level of analyte determined after the third cycle was within 3% of the initial value for plasma samples spiked at the 1.8 and 178 ng/ml I levels.

3.5. Stability of I in LLE extracts

In addition to stability in the sample matrix, the stability of I in the hexane extract was examined to determine the timing required for the actual sample analysis. Standards and spiked samples, dog and rat plasma matrix, were prepared and analyzed on the same day, after sitting on the autosampler for 16 h at ambient temperature and after refrigerated storage for 5 days (Table 3). Compound I was found to be stable in the LLE extracts, both overnight at ambient temperature and after storage in the refrigerator for 5 days.

3.6. Pharmacokinetic analysis for rat and dog

Plasma profiles obtained following intravenous administration of I to rats and dogs are shown in Fig. 4. The pharmacokinetic parameters are presented in Table 4. The decline of I plasma levels displays a distinct polyexponential behavior with a 3.5–4-log decline in plasma levels within 24 h after dosing. Though the terminal half-life of I was >44 h in rats and dogs, this represents a relatively small percentage, <5%, of the total administered dose. Volumes of distribution were quite large in both rat and dog, indicating the lipophilic nature of the compound.

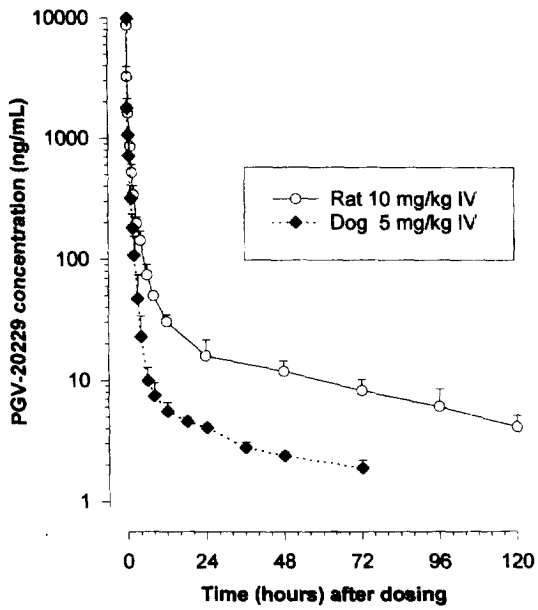

Fig. 4. Mean plasma levels of I following intravenous administration to beagle dogs (5 mg/kg I) and Sprague–Dawley rats (10 mg/kg I). Data represent mean and standard deviation ($n=3$ dogs per observation; $n=6$ rats per observation).

Table 3
Stability of I in hexane extract (drug analysis after storage at ambient temperature for 16 h and refrigerated for 5 days)

Spiked [I] (ng/ml)	Percent of Initial analysis			
	Ambient storage		Refrigerated	
	Rat	Dog	Rat	Dog
1.8	95	101	107	98
18	99	101	104	101
178	98	99	96	97
1780	104	99	103	108

4. Conclusion

A selective and sensitive GC–MS method was developed for the analysis of a novel antiinflammatory agent, I, in small volumes of rat and dog plasma. The sensitivity of the method allowed plasma levels of the parent drug to be determined for a sufficiently long time period to accurately define the terminal half-life for this lipophilic compound.

Table 4
Pharmacokinetic parameters for I administered to rats and dogs

Species	Dose i.v. (mg/kg)	Cl (ml/min/kg)	V_z (l/kg)	V_{ss} (l/kg)	$T_{1/2}$ (h)	K_e (h^{-1})	MRT (h)
Rat	10	30.0±3.6	127.6±25.8	39.2±13.2	48.7±5.6	0.014±0.002	21.7±6.3
Dog	5	23.7±6.8	92.1±33.8	13.5±7.8	44.9±13.8	0.016±0.005	9.5±6

Data represent mean values for six rats and three dogs. Cl, clearance; V_z, apparent volume of distribution; V_{ss}, volume of distribution steady state; $T_{1/2}$, terminal elimination half-life; K_e, elimination rate constant; and MRT, mean residence time.

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