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Development and validation of a liquid chromatography–mass spectrometric method for the determination of DPC 423, an antithrombotic agent, in rat and dog plasma

Cecilia Chi[a,*], Li Liang[a], Patty Padovani[b], Steve Unger[c]

[a]*Bristol-Myers Squibb Company, Metabolism & Pharmacokinetics, PRI, Experimental Station, Rt 141 and Henry Clay Rd., Wilmington, DE 19803-0353, USA*

[b]*Astrozeneca LP, 1800 Concord Pike, Wilmington, DE 19803, USA*

[c]*Bristol-Myers Squibb Company, Building 105, Office 2465, P.O. Box 191, New Brunswick, NJ 08903, USA*

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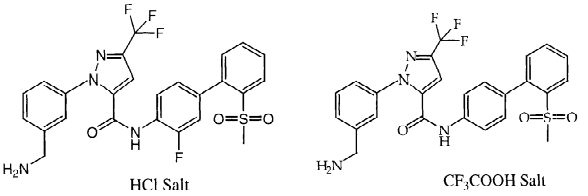
Abstract

A sensitive and selective LC–MS–MS method for the determination of DPC 423 (**I**), an antithrombotic agent, is described. This method used a solid-phase extraction from 0.1 ml plasma with an Isolute C_2 cartridge. HPLC separation was carried out on a YMC ODS-AQ C_{18} column (50×2 mm) at a flow-rate of 300 μl/min with an analysis time of 5 min. Compounds were eluted using a mobile phase of $H_2O/CH_3CN/HCOOH$: 66:34:0.1 (v/v/v), pH 4.0. A structural analogue of **I** was used as the internal standard to account for variations in recovery and instrument response. Mass spectrometric detection was carried out with a PE Sciex API III$^+$ triple quadrupole mass spectrometer equipped with a Turbo IonSpray™ source as the LC–MS interface. Good intra-day and inter-day assay precision (<10% CV) and accuracy (<10% difference) were observed over a concentration range of 0.005–2.5 μ*M* in plasma. The extraction recoveries were approximately 90% and the method was found to be linear for the assay (r^2>0.999). The method has been successfully applied to discovery and preclinical pharmacokinetic studies, including a dose range-finding study and toxicokinetic exposure studies in rat and dog.

Keywords: Antithrombotic agents; DPC 423

1. Introduction

DPC 423 (**I**), 1-(3-aminomethylphenyl)-5-[(3-fluoro-2′-methylsulfonyl-[1,1′]-biphen-4-yl) amino-carbonyl]-3-trifluoromethyl pyrazole, hydrochloric acid salt (Fig. 1) is an inhibitor of coagulation factor Xa with high affinity and selectivity [1]. Several



DPC 423 (I) **SS456 (IS)**

Fig. 1. Structures of **I** and **IS**.

*Corresponding author. Tel.: +1-302-467-5209; fax: +1-302-467-6712.

E-mail address: cecilia.chi@bms.com (C. Chi).

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potent inhibitors of factor Xa have been published [1–9]. Venous and arterial thromboembolism are common medical disorders in need of a more efficacious, convenient, and safer therapeutic agent. In the United States, mortality related to cardiovascular diseases approaches one million people per year, with a high proportion resulting as a consequence of thrombotic processes. Current available treatment for venous thrombosis and embolic diseases include anticoagulants (warfarin and other coumarin derivatives, heparin, and low molecular mass heparin) which although effective, have either a narrow therapeutic index (i.e. warfarin) or require administration by injection (i.e. heparin, low molecular mass heparin) [1,3,5]. Among potential targets for improving the treatment of thromboembolic diseases is the inhibition of factor Xa activity. Factor Xa plays a pivotal role in the coagulation cascade, linking the intrinsic and the extrinsic systems of coagulation. It is anticipated that inhibitors of factor Xa will have a more favorable efficacy to safety ratio than will other antithrombotic agents [1–5].

In order to investigate the bioavailability and the pharmacokinetics of **I** in rats and dogs, an LC–MS–MS assay was developed. There are several analytical methods reported for the quantitation of anticoagulant and antithrombotic agents in biological fluid [10–16]. LC–MS–MS has been increasingly used to perform bioanalytical determinations with maximum selectivity, sensitivity, and throughput. The application of LC–MS–MS is the technique that is currently considered the method of choice for supporting clinical and preclinical pharmacokinetic studies [17–21]. We have been using LC–MS–MS to generate both in vivo and in vitro ADME (Absorption, Distribution, Metabolism and Elimination) data in the drug discovery process. Along with a positive biological control, compounds are dosed as mixtures and their pharmacokinetic properties are assessed. Provided that the control compound responds appropriately, new compounds with the best pharmacokinetic and pharmacological properties are selected for progression as drug candidates [22–26]. Chemical diversity is an important component in pool selection. A non-selective extraction, acetonitrile protein precipitation, and gradient HPLC separation were used due to the wide variety of physical and chemical properties of the compounds. Based upon the pharmacology, toxicology, and pharmacokinetics, **I** was chosen as the optimum drug candidate for development [9]. This paper describes the development, validation and application of a specific LC–MS–MS procedure to support its drug development in rats and dogs.

2. Experimental

2.1. Reagents, chemicals and biological matrices

DPC 423 (**I**) and SS456 (**IS**) (Fig. 1) were synthesized by Bristol-Myers Squibb Company (Wilmington, DE, USA). Acetonitrile (HPLC grade), formic acid (ACS grade), methanol (HPLC grade), ammonium hydroxide (HPLC grade) and semicarbazide were purchased from EM Science (Gibbstown, NJ, USA). HPLC quality water was prepared using a Millipore Milli-Q purification system (Millipore, Milford, MA, USA). Sprague–Dawley rat plasma and beagle dog plasma with sodium citrate as the anticoagulant were purchased from Cocalico (Reamstown, PA, USA). Isolute™ C_2 100 mg/1 ml solid-phase cartridges were purchased from International Sorbent Technology (Mid-Glamorgan, UK).

2.2. Apparatus

The HPLC system consisted of a HP series 1100 solvent delivery system (Waldbronn, Germany) and a Waters 717+ autosampler (Medford, MA, USA). Mass spectrometric detection was carried out using a PE Sciex API III$^+$ triple quadrupole instrument (PE-Sciex, Thornhill, Canada) equipped with a Turbo IonSpray™ interface. A Mac QUADRA 950 computer from Apple MacIntosh (Austin, TX, USA) equipped with Sciex RAD version 2.6 and MacQuan version 1.4 software from PE Sciex was used to collect and to process data.

2.3. Chromatographic conditions

HPLC separation was carried out on a YMC ODS-AQ C_{18} column (50×2 mm) at a flow-rate of 300 μl/min with an analysis time of 5 min, operated at ambient temperature. Compounds were eluted using

Table 1
Mass spectra intensity (%)

Compound	m/z	Intensity (%)
I	498	100
	437	44
	516	19
IS	480	100
	498	50
	419	13

a mobile phase of $H_2O/CH_3CN/HCOOH$: 66:34:0.1 (v/v/v), pH 4.0.

2.4. Mass spectrometric conditions

Multiple reaction monitoring (MRM) in the positive ionization mode was carried out. The first quadrupole, Q1, selected the protonated molecules $(M+H)^+$, at m/z 533 for **I** and m/z 515 for **IS**. The product ions (m/z 498 for analyte and m/z 480 for **IS**) were generated by collision-induced fragmentation within Q2 (collision gas argon, 250×10^{12} atoms/cm^3) and detected at the electron multiplier. These product ions were chosen based on their significance in the MS–MS spectra. The product ion mass spectrum of the $(M+H)^+$ ion of **I** showed intense fragments at m/z 498 $[(M+H)^+–NH_3–H_2O]$, m/z 437 $[(M+H)^+–NH_3–SO_2CH_3]$ and m/z 516 $[(M+H)^+–NH_3]$. The **IS** gave fragments at m/z 480 $[(M+H)^+–NH_3–H_2O]$, m/z 498 $[(M+H)^+–NH_3]$ and m/z 419 $[(M+H)^+–NH_3–SO_2CH_3]$ (Table 1). MRM using the precursor→product ion combinations at m/z 533→498 for DPC 423 and m/z 515→480 for **IS** allowed highly sensitive detection of the analyte. There is always the risk of reducing selectively by choosing simple neutral losses in the case of the transition m/z 533→498 for **I**. However, the results obtained from monitoring a second transition for **I** (533→437) demonstrated that selectivity was maintained (Table 2).

Turbo IonSpray temperature was maintained at 450 °C. The nebulizing gas (nitrogen) pressure and auxiliary gas (nitrogen) flow were at 64 p.s.i. and 6.5 l/min, respectively. Curtain gas (nitrogen) flow-rate was 1.2 l/min at 60 p.s.i and the temperature of the interface heater was set at 60 °C. The ion spray and orifice voltages were set at 4890 and 65 V, respectively. The mass spectrometer was operated with unit resolution for both Q1 and Q3 (e.q. 0.7 Da at 50% height).

Data were acquired with a dwell time of 200 ms, a pause time of 0.02 ms, a scan rate of 2.17/s and the count controller (CC) was set to 1. Peak area ratio were used for calculation and the calibration curve was fitted to a weighted $1/X^2$ linear regression model using PE Sciex software MacQuan version 1.4 with no smoothing applied. Concentrations for unknowns were determined from the equation:

$$\text{Conc.} = \left(\frac{\text{PAR} - (y\text{-intercept})}{\text{Slope}}\right)\left(\frac{0.1\ \text{ml}}{\text{Sample volume}}\right)$$

where PAR is the peak-area-ratio of analyte to **IS**.

2.5. Sample preparation

2.5.1. Preparation of solution

Primary stock solutions of **I** and internal standard (**IS**) were prepared by dissolving accurately weighed quantities of **I** and **IS** in methanol at nominal concentrations of 1.0 mM and 100 μg/ml, respectively. Working stock solutions of **I** over the concentration range of 0.02–10 μM were prepared by serial dilution with methanol from the primary stock solution. The working stock solution of internal

Table 2
The experiments demonstrated that by monitoring two fragmentations, selectivity was maintained

Nominal concentrations (μM)	m/z 533→498 Mean found concentrations (μM)[a]	m/z 533→437 Mean found concentrations (μM)[a]	% Different
0.00500	0.00498	0.00476	−4.42
0.1250	0.1250	0.1249	−0.09
2.500	2.475	2.492	0.69

[a] All values are expressed as the mean of three determinations.

standard (1 μg/ml) was prepared from the primary stock of 100 μg/ml. All primary and working stock standard solutions were stored at −20 °C and were stable for at least 5 months (Table 3). HPLC mobile phase was prepared by combining 340 ml of acetonitrile and 660 ml of water and 1 ml of formic acid. Combining 500 ml of methanol and 500 ml of water and 1 ml of formic acid prepared reconstitution solution. The pH of mobile phase and reconstitution solution was adjusted to 4.0 with ammonium hydroxide.

2.5.2. Extraction procedure

Standard and QC samples were prepared by adding 10 μl of semicarbazide (5 m*M*) and 100 μl of rat (or dog) plasma into a clean 12×75 mm glass test-tube. Standard and QC samples were spiked with 25 μl of **I** standard working solution, while study samples were spiked with an equivalent volume of methanol. Semicarbazide was added to the study samples while samples were collected. Semicarbazide was added to prevent the loss of plasma enzyme catalyzed deamination of **I** [27]. A 25-μl aliquot of internal standard working solution and 1 ml of water were added to all tubes and vortexed briefly. The final concentrations of **I** standards in plasma were 0.005, 0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25, and 2.5 μ*M*. The sample was transferred to an Isolute™ C_2 solid-phase extraction cartridge that had been conditioned sequentially with 1 ml of methanol and 1 ml of water. The column bed was then rinsed twice with 1 ml of 10% methanol in water. After vacuum drying, **I** and **IS** were eluted with two 0.5-ml aliquots of methanol into a 12×75 mm glass test-tube. All solvents and samples were eluted from SPE columns by gravity. The eluant was dried under nitrogen at 37 °C and the residue was reconstituted with 0.2 ml of reconstitution solution, vortexed briefly and transferred to an auto-sampler vial for LC–MS–MS analysis. To avoid loss due to adsorption, sample reconstitution was performed within 1 h after drying.

2.6. Application

An application of the method for pharmacokinetic studies was demonstrated in the analysis of plasma samples from rats and dogs dosed with **I**. After overnight fasting, Sprague–Dawley CD rats ($n=3$) and beagle dogs ($n=4$) received an i.v. single dose

Table 3
Stability of **I** stock solution and plasma control samples

Nominal concentrations	Rat (n*M*)		Dog (n*M*)	
	50	250	50	250
Plasma sample ($n=3$)				
24 h, 20 °C				
found concentrations (nM)	49.1	240.9	48.0	259.3
% of initial	101.1	100.2	97.1	101.2
Plasma sample storage ($n=3$);				
rat 54 days, dog 59 days, −20 °C				
found concentrations (nM)	47.0	236.4	38.5	192.8
% of initial	98.3	98.6	88.9	88.1
Freeze–thaw ($n=3$)				
1 cycle (% of initial)	89.5	94.8	100.9	104.1
2 cycle (% of initial)	109.5	86.9	117.9	100.5
3 cycle (% of initial)	97.7	96.1	109.9	105.2

	Stock solution (n*M*)	
	100	5000
Stock solution ($n=3$)		
5 months, −20 °C		
% of initial	99.7	105.9

at 5 mg/kg and 2 mg/kg, respectively. Blood samples were collected in citrated Vacutainers® by jugular venipuncture up to 24 h postdose. The blood was centrifuged and the plasma was transferred to tubes containing semicarbazide. Samples were kept frozen at −20 °C until assayed for **I** concentration.

3. Results and discussion

3.1. Selection of extraction and chromatographic systems

Different types of SPE (solid-phase extraction) cartridges containing bonded silicas with various chemical properties were tested. The cartridges tested were C_{18}, C_{18} (end-capped), C_8, C_8 (end-capped), C_2, C_2 (end-capped), from IST (International Sorbent Technology) development kit, Waters OASIS C_{18}, and Varian C_2. All cartridges were sorbent mass 100 mg and reservoir volume 1 ml. The extraction recoveries of **I** were determined. Varian C_2 and IST C_2 had the best extraction recovery among all cartridges. However, the samples extracted from the Varian C_2 were not clean, and clogged the analytical column. The Isolute™ C_2 SPE cartridge from IST was chosen.

Potential wash solvents were evaluated by increasing methanol concentrations to determine the strongest wash solvent which did not elute **I** or **IS**. The 10% methanol aqueous solution was chosen as the wash solution because it resulted in chromatograms that were free of interference and did not elute **I** or **IS**.

We tested the chromatographic behavior of **I** on a number of columns. These columns included YMC ODS-AQ 50×2 mm C_{18}, MetaChem 50×2 mm C_{18}, and SMT 50×2.1 mm C_{18}. In most cases, the resolution was not satisfactory because of broad and asymmetrical peaks. The YMC ODS-AQ 50×2 mm column was chosen because it resulted in the best peak shape for both **I** and **IS**.

3.2. Linearity and specificity

The calibration curves were linear over the concentration range of 0.005–2.5 μ*M* in rat and dog plasma with a mean correlation coefficient (r^2) of >0.999. Standards concentrations were back-calculated and the relative concentration residuals (RCRs) were calculated as the equation below:

$$\%RCR = (FC - NC)/NC \times 100$$

where FC is the found concentration and NC is the nominal concentration.

The RCR values at all levels were less than 15% and deemed acceptable according to our SOP. To evaluate the assay specificity, six independent lots of rat and dog control plasma were used. Assay specificity was demonstrated by the absence of interfering peaks at the retention time of **I** and **IS** in the blank rat and dog plasma samples. The limit of detection (LOD) in plasma was defined by the concentration with a signal-to-noise ratio of 3. The LOD in both rat and dog plasma were about 0.0025 μ*M* for DPC 423. The retention times for both **I** and **IS** were approximately 3.0 min (Figs. 2 and 3).

3.3. Stability

Stability samples of **I** in frozen plasma were analyzed periodically in triplicate at concentrations of 0.05 and 0.25 μ*M*. The stability of **I** in rat and

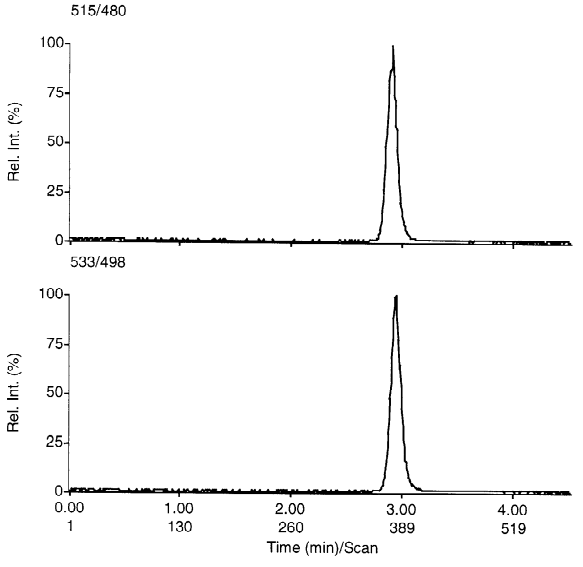

Fig. 2. Representative chromatograms of rat plasma extracts 1 h after a 5 mg/kg i.v. dose of **I**; plasma found concentration 0.623 μ*M* (lower panel: **I**, 533/498; upper panel: **IS**, 515/480).

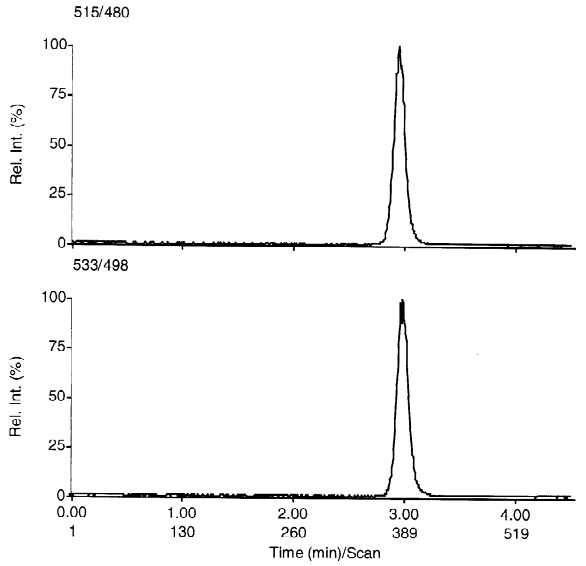

Fig. 3. Representative chromatograms of dog plasma extracts 5 h after a 2 mg/kg i.v. dose of **I**; plasma found concentration 0.540 μ*M* (lower panel: **I**, 533/498; upper panel: **IS**, 515/480).

dog plasma, stored frozen at −20 °C for at least 2 months, was demonstrated (Table 3). No significant change was detected in **I** in rat and dog plasma stored at room temperature for 24 h and after three freeze–thaw cycles (Table 3).

3.4. Assay precision and accuracy

The precision of the assay was defined as the coefficient of variation (%CV) calculated from replicate measurements. The accuracy of the assay was defined as the mean of the absolute values of the percent difference of the determined concentrations from the nominal value [28]. The percent coefficient of variation (%CV) and the percent difference for each measurement are determined by:

$$\%\mathrm{CV} = \mathrm{SD}/X \times 100$$

where SD is the standard deviation $\sigma(n-1)$, and X is the mean.

$$\%\mathrm{Difference} = \left[\frac{\text{determined} - \text{nominal}}{\text{nominal}}\right] \times 100$$

Seven concentrations of control samples, prepared independent of the analyst, were analyzed for intra-day and inter-day reproducibility. Five replicates were prepared at each concentration and analyzed on the same run for intra-day precision and accuracy. Inter-day assay precision and accuracy were determined on three different days. The results for the intra-day and inter-day precision and accuracy are shown in Tables 4 and 5. In both rat and dog plasma, the intra-day and inter-day precision and accuracy of the method was acceptable and was not dependent upon concentration.

3.5. Extraction recovery, matrix effect and carry-over

Mass spectrometric detection, while very selective and sensitive, may be adversely affected by ion suppression (or enhancement) caused by the other components within the sample matrix. A number of reports have demonstrated that matrix effect plays an important role in LC–MS–MS method development and application [29–34]. The evaluation and elimination of matrix effects should be a part of any method development for MS-based assays. While higher resolution mass analyzers and more selective or extensive fragmentation can serve to reduce chemical noise, detection is employed following ion formation and, therefore, cannot correct for competitive ioniza-

Table 4
Intra-day assay precision and accuracy from rat and dog plasma ($n=5$)

Nominal plasma concentration (μ*M*)	Mean found concentration (μ*M*)	CV (%)	% Difference
Rat			
0.0050	0.0052	4.3	4.7
0.0125	0.0126	4.2	3.2
0.0250	0.0248	3.1	2.4
0.125	0.118	3.3	5.5
0.500	0.491	0.43	1.7
1.250	1.185	2.5	5.2
2.500	2.395	4.1	4.2
Dog			
0.0050	0.0050	7.9	5.9
0.0125	0.0119	3.3	5.2
0.0250	0.0248	4.0	3.3
0.125	0.121	1.9	3.4
0.500	0.465	6.5	7.1
1.250	1.216	3.3	2.7
2.500	2.523	1.2	1.2

Table 5
Inter-day assay precision and accuracy from rat and dog plasma ($n=3$)

Nominal plasma concentration (μM)	Mean found concentration (μM)	CV (%)	% Difference
Rat			
0.0050	0.0048	6.0	4.7
0.0125	0.0132	7.2	7.0
0.0250	0.0227	0.56	0.96
0.125	0.118	6.7	5.6
0.500	0.466	7.7	6.8
1.250	1.183	6.5	5.4
2.500	2.368	8.6	5.3
Dog			
0.0050	0.0054	9.9	11.0
0.0125	0.0123	9.2	7.5
0.0250	0.0258	1.9	3.2
0.125	0.126	3.0	2.0
0.500	0.479	12.0	7.1
1.250	1.233	6.0	4.1
2.500	2.522	2.6	1.7

tion effects. The development of separation processes, both extraction and chromatographic, must consider matrix effects. With high speed chromatographic separations, most bioanalytical applications should employ a peak capacity factor (k') of at least three to avoid common ionic interferences that suppress ESI response. A simple adjustment of the proportion of organics within the mobile phase often achieves the desired result.

A set of **I** extracted QC samples (in matrix), a set of unextracted QC samples (in reconstitution solution, no matrix) and a set of post-extracted spiked QC samples (extracted blank sample reconstituted with unextracted QC) were analyzed on the same run to determine the extraction recovery and matrix effect.

The extraction recovery (or extraction efficiency) was determined by measuring an extracted sample against a post-extraction spiked sample:

$$\text{Extraction recovery} = \left(\frac{\text{Response of extracted sample}}{\text{Response of post-extracted spiked sample}}\right)\times 100\%$$

The matrix effect was measured by referring the post-extracted spiked sample to the unextracted sample:

$$\text{Matrix effect} = \left(\frac{\text{Response of post-extracted spiked sample}}{\text{Response of unextracted sample}}\right)$$

The absence of a matrix effect is indicated by a ratio of 1.0. No response due to total matrix suppression would give a value of 0.

After comparing the response of the extracted QC samples with that of the post-extracted sample and comparing the response post-extracted versus that of unextracted, the extraction efficiency and matrix effect in rat and dog plasma were determined (Tables 6 and 7). The extraction recovery of **I** and **IS** from 0.1 ml of rat and dog plasma appeared independent

Table 6
I and **IS** rat plasma matrix effect and extraction efficiency

	I concentration (μM)	Unextracted samples (peak area)[a]	Post-extracted spiked samples (peak area)[a]	Extracted samples (peak area)[a]	Matrix effect	Extraction efficiency (%)
I	0.005	1195	1051	906	0.88	86.2
	0.125	32 646	25 185	21 584	0.77	85.7
	2.500	585 056	485 775	457 114	0.83	94.1
	Mean				0.83	88.7
	SD				0.05	4.7
IS	0.005	126 740	105 791	90 240	0.83	85.3
	0.125	126 964	105 949	97 579	0.83	92.1
	2.500	110 302	91 857	85 060	0.83	92.6
	Mean				0.83	90.0
	SD				0.00	4.1

[a] All values are expressed as the mean of five determinations.

Table 7
I and **IS** dog plasma matrix effect and extraction efficiency

	I concentration (μM)	Unextracted samples (peak area)[a]	Post-extracted spiked samples (peak area)[a]	Extracted samples (peak area)[a]	Matrix effect	Extraction efficiency (%)
I	0.005	1195	1002	847	0.84	84.5
	0.125	32 646	25 847	20 626	0.79	79.8
	2.500	585 056	483 280	425 286	0.83	88.0
	Mean				0.82	84.1
	SD				0.02	4.1
IS	0.005	126 740	105 371	90 303	0.83	85.7
	0.125	126 964	95 045	81 454	0.75	85.7
	2.500	110 302	91 805	78 493	0.83	85.5
	Mean				0.80	85.6
	SD				0.05	0.01

[a] All values are expressed as the mean of five determinations.

of concentration within the range of plasma concentrations studied.

Slight ion suppression was observed in the assay. The extent of suppression on the analyte and internal standard was about the same. The **IS** was chosen to be a close structural analogue whose ion formation and fragmentation were similar to **I**. To ensure that any changes in analyte response were compensated, chromatographic conditions were chosen to co-elute the **IS** with the analyte.

Carry-over was examined by alternately analyzing blank extracted plasma samples and extracted plasma sample containing concentrations at the ULOQ (2.5 μM). No carry-over was observed.

3.6. Adsorption

Adsorption was one of the problems encountered during the assay development. Both drug and internal standard are readily adsorbed onto glass surfaces. An experiment was carried out in which both drug and internal standard solution were spiked into a borosilicate glass tube, dried under nitrogen, and kept dry for different periods of time at 22 and 37 °C, respectively. There was no significant difference at either temperature in the loss of drug and internal standard to glass surface, suggesting the loss was most likely due to adsorption and not thermal degradation (Fig. 4). The extent of adsorption onto glass surfaces for **I** and **IS** was about the same. The structural analogue internal standard minimizes differences between the analyte and internal standard, giving a constant ratio. The experiment further demonstrated that reconstituting the extracted sample within 1 h could avoid the loss due to adsorption.

3.7. Application

The method has been used successfully to evaluate the pharmacokinetics of **I** in rats and dogs. Representative plasma concentration versus time profiles are shown for rat and dog in Fig. 5. Following administration of an i.v. dose of 5 and 2 mg/kg in rats ($n=3$) and dogs ($n=4$), respectively, **I** exhibited a polyexponential decline in plasma concentrations

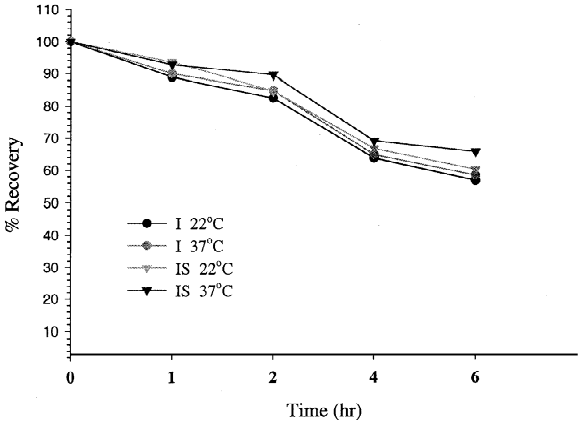

Fig. 4. Percent recovery of unextracted **I** and **IS** after incubating at 22 or 37 °C for 0–6 h in glass tubes.

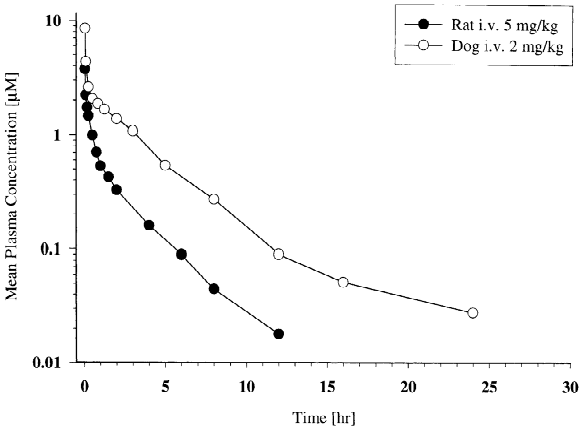

Fig. 5. Representative mean plasma concentrations versus time profiles after i.v. administration of **I** in rat and dog.

in both animal species. The pharmacokinetic parameters are shown in Table 8. The concentrations in all samples were well within the range of the standard curves (0.005–2.5 μ*M*), indicating that this method is suitable for pharmacokinetics evaluation in rat and dog.

An assay to measure **I** in human plasma has also been developed and validated in the range of 0.2–500 n*M* using 0.2 ml plasma and the method has been used successfully in human clinical studies. Semicarbazide was not added to human plasma to prevent enzyme-catalyzed deamination of **I** [27]. A plasma stability study of **I** demonstrated that the plasma samples from different species with **I** added showed species-dependent loss of the compound. The plasma of rhesus monkey, rabbit, and dog showed rapid degradation and needed semicarbazide to stabilize **I**. The plasma of rat, chimpanzee, and human did not need semicarbazide to prevent the loss of the compound. However, in order to unify the animal assay procedure, semicarbazide was added to both rat and dog plasma during the sample preparation.

Table 8
Pharmacokinetic parameters

Parameter	Rat (i.v., 5 mg/kg, $n=3$)	Dog (i.v., 2 mg/kg, $n=4$)
$t_{1/2}$ (h)	2.6±0.3	7.2±1.4
Vdss (l/kg)	4.4±0.3	0.9±0.2
CL (l/h per kg)	1.8±0.2	0.2±0.0

4. Conclusion

An LC–MS–MS based assay for **I** has been developed and validated over the concentration range of 0.005–2.5 μ*M* in rat and dog plasma. The assay was precise, accurate, and robust (Tables 4 and 5, criteria: %CV<15%, %diff<15%). A close structural analogue was used as the internal standard to account for variations due to adsorption, matrix effect, extraction, and instrument performance. Reconstituting samples within 1 h after evaporation of SPE elution solvent was carried out to overcome adsorption losses. Extraction of control plasma demonstrated no interference from endogenous substances. The method has been successfully used in determining **I** in rat and dog plasma for pharmacokinetic and drug development safety studies.

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