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Determination of a specific 5-HT$_{2a}$ antagonist (M100907) in rat and dog plasma at the femtomole/milliliter level by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric method for the determination of *R*(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)-4-piperdinemethanol] (M100907), **I**, in rat and dog plasma is described. **I**, a specific 5-HT$_{2a}$ receptor antagonist and the internal standard were concentrated from plasma by C_1 solid-phase extraction. Following derivatization to their TMS ethers, they were separated by capillary GC and detection was by mass specific detection in single-ion monitoring mode. The validation range was from 0.1 to 10 ng/ml. The day-to-day coefficient of variation for the calibration standards over the concentration range varied from 9.5 to 14.9% in dog plasma and 1.7 to 13.4% in rat plasma. Quality control standards were measured at three concentrations (0.5, 2.5 and 7.5 ng/ml) in plasma from both species; the day-to-day coefficient of variation ranged from 1.8 to 14.2% in dog plasma and 1.6 to 15.1% in rat plasma. The method is both specific and sensitive down to the picomolar level in plasma. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: M100907; 5-Hydroxytryptamine; Schizophrenia; Atypical; Antipsychotic

1. Introduction

Schizophrenia is an episodic disease in which the symptoms are generally believed to be due to an overac active dopaminergic system in the limbic forebrain [1]. All currently used antipsychotic drugs have dopamine blockade as a component of their pharmacological profile. The result of global attenuation of the dopaminergic system is improvement in symptomatic control of psychoses along with a number of potentially severe side effects (e.g., parkinsonism, bradyphrenia, dysphoria, etc.). *R*(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)-4-piperdinemethanol] (M100907), (**I**, Fig. 1 is an orally active, highly selective and potent 5-HT$_{2a}$ antagonist that may provide the first non-dopaminergic approach to the treatment of schizophrenia [2]. A unique feature of this compound is its

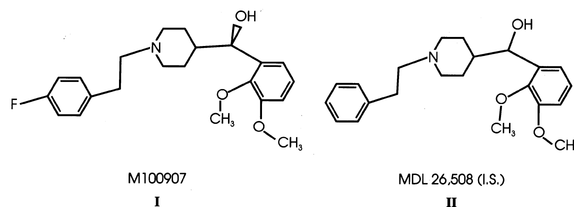

Fig. 1. Chemical structures of M100907 (**I**) and the internal standard, MDL 26,508 (**II**).

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low affinity for other neurotransmitter receptors, including dopamine D2 receptors, alpha-1 receptors, $5HT_{2c}$ receptors, cholinergic M1/M2 receptors. Because of its high selectivity, **I** may be devoid of many of the side effects related to nonspecificity of the traditional antipsychotics and the newer nonselective multireceptor antagonists such as clozapine, risperidone and olanzapine which include sedation, weight gain, hypotension and cognitive deficits [3].

The paper describes the development and validation of an assay for **I** that involves solid-phase extraction of the analyte and internal standard from plasma followed by derivatization to their trimethylsilyl ethers, separation by capillary gas chromatography and detection by mass spectrometry. This assay was used to support pharmacokinetic and toxicokinetic studies in rat and dog.

2. Experimental

2.1. Materials

R(+)-α-(2,3-dimethoxphenyl)-1-[2-(4-fluorophenylethyl)-4-piperdinemthanol], [M100907 (**I**)] and α-(2,3-dimethoxphenyl)-1-[2-(phenylethyl)-4-piperdinemthanol]-HCl, [MDL 26,508, (**II**)] were synthesized at Marion Merrell Dow Research Institute (Cincinnati, OH, USA).

All organic solvents were HPLC grade and were from Burdick and Jackson (Baxter, Muskegon, MI, USA). Pyridine was high purity, also from Burdick and Jackson. Bis(trimethylsilyl)trifluroracetamide (BSTFA) was from Regis (St. Louis, MO, USA). Reconstitution solvent was *n*-tridecane obtained from Sigma Chemical (St. Louis, MO, USA). Water was HPLC grade obtained from Mallinckrodt (Paris, KY, USA). Heparinized dog and rat plasmas were obtained from Cocalico Biologicals (Reamstown, PA, USA). C_1 cartridges (1-ml capacity) were purchased from Varian–Anayltichem International (Harbor City, CA, USA).

2.2. Chromatographic conditions

The chromatographic system consisted of a gas chromatograph (GC)/mass selective detector (MSD) from Hewlett Packard (Model 5890, series II; Palo Alto, CA, USA) equipped with a DB 5 fused-silica capillary column (0.4-mm film thickness, 0.18 mm× 20 m; J and W Scientific, Folsum, CA, USA). The GC column oven program was: 200°C initial hold for 1 min followed by a 20°C/min increase to 350°C and maintained at this temperature for 4 min. The injector oven temperature was set to 320°C, the transfer line oven was set to 280°C. Splitless injections were made for 45 to 75 s. Injection volume was 2 μl and GC column flow (helium) was kept constant at 1.2 ml/min. The MSD detector was used in electron impact mode (70 eV) with a 5-min solvent delay. In the selected-ion monitoring (SIM) mode, the ion monitored was m/z 336 with a dwell time of 400 ms.

2.3. Standard solutions

Stock solutions of **I** (0.402 mg/ml) and **II** (0.907 mg/ml) were prepared in methanol and stored at 4°C. Working solutions were obtained by dilution of the stock solution with methanol to yield concentrations of 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml for **I** and 0.125 mg/ml for **II**. Solutions were stable for at least 6 months at 4°C.

2.4. Calibration standards and quality control standards

For each calibration curve, duplicate samples of either rat or dog plasma (0.5 ml each) were fortified with **I** at concentrations of 0, 0.1, 0.25, 0.5, 1 and 10 ng/ml. The total volume of methanol added was 50 μl. The concentration of **II**, the internal standard was set at 2.3 ng/ml of the free base. Quality control standards were prepared at four concentrations (0.15, 0.5, 2.5 and 7.5 ng/ml). Separate quality control standards (pools of 50 ml) were prepared in plasma from each species, 0.5-ml aliquots were transferred into 2-dram vials and stored at −20°C.

2.5. Sample preparation

To 0.5 ml of plasma (either rat or dog) was added, 20 μl of internal standard solution and 50 μl of methanol. Samples were well mixed before solid-phase extraction. Extraction was achieved using the C_1 1-ml cartridges, conditioned with methanol (2

ml) followed by water (2 ml). After transferring the samples to the cartridges, the solvent was eluted under a slight vacuum (~5 mm Hg). The bed was washed twice with water (1 ml each) and the cartridges were allowed to dry under a slight vacuum for ~5 min. Analytes of interest were then eluted from the cartridge with two 1-ml portions of methanol and collected in screw cap round bottom tubes (13×100 mm). The contents of each tube were reduced to dryness using a Savant Speedvac, with a minimum drying time of 1.5 h.

Two different but equivalent procedures were used for derivatization. For rat plasma samples, the residue from the SPE step was reconstituted in BSTFA (190 ml), capped and heated for 30 min at 60°C. Residues from the SPE step for dog plasma samples were reconstituted in a mixture (200 μl) of ethyl acetate–pyridine–BSTFA (1:1:2, v/v), capped and heated for 30 min at 60°C. After derivatization of the analytes to their trimethylsilyl (TMS) ethers, the contents were transferred to GC autosampler vials containing 200 μl inserts and brought to dryness in the SpeedVac (30 min). A mixture of BSTFA and tridecane (50 μl) in a 1:1 v/v ratio was added to the inserts prior to capping. Samples were mixed and analyzed by GC–MS.

2.6. Extraction efficiency

The extraction efficiencies at two concentrations of **I** (0.5 and 5 ng/ml in rat plasma and 0.5 and 10 ng/ml in dog plasma) and a single concentration of **II** (4.6 ng/ml in both species) were determined in duplicate.

2.7. Data analysis

Peak integration was performed by Hewlett Packard Chemstation software. Peak areas were than entered into a regression and statistical analysis program (BA_STAT) which calculated the line of best fit for calibration standards using a weighted ($1/x$) quadratic least-squares regression of analyte to internal standard peak area ratios Concentrations of **I** in quality control standards and study samples were calculated by interpolation from the line of best fit for the calibration standards and corrected for dilution if needed.

2.8. Assay validation

The accuracy and precision of the method were determined by the replicate analysis of pools of control plasma fortified with **I** at levels of 0.15, 0.5, 2.5 and 7.5 ng/ml. These levels were chosen to demonstrate the accuracy and precision of the method at or near the lower limit of the calibration curve as well as in the low, middle and high ranges of the curve. The analyses of 12 samples at each concentration (except for the 0.15 ng/ml concentration in rat plasma) were conducted in three separate batches. Three replicates for each concentration were analyzed during two of the batches and six replicates of each concentration during one of the batches. This arrangement allowed for the determination of within-day and between day precision of the method.

A set of calibration curve standards, five concentrations performed in duplicate for each batch, were analyzed for both rat and dog plasma. Comparison of back-calculated calibration curve standards to the calculated line provides another view of the accuracy and precision of the method. The percent relative error, R.E. (%), is used as a measure of accuracy and is defined as: [observed/theoretical−1]×100. A measure of the precision is the percent relative standard deviation, R.S.D. (%) or (standard deviation/mean)×100.

2.9. Solution and storage stability

The stability of **I** in the stock solutions, injection solvent and plasma was assessed. The stability of the drug in the injection solvent was assessed by repeat analysis of a set of calibration and quality control standards after storing for 4/5 days at room temperature.Stability of **I** stored in plasma (either dog or rat) at −20°C was demonstrated through the analysis of quality control standards at concentrations of 0.5 and 7.5 ng/ml ($n=3$ for each per day) on days 46 in dog and 77 in rat plasma. These are the same quality control pools as those used in the three days of validation. Also the effect of three freeze–thaw cycles on **I** in both rat and dog plasma at con-

centrations of 0.5 and 7.5 ng/ml (n=3) was assessed.

3. Results and discussion

Measurement of concentrations of **I** in rat or dog plasma consists of isolation of the drug and **II** by solid-phase extraction, evaporation of solvent, derivatization of the analytes to their TMS ethers, separation of the analytes by capillary GC, and detection by MSD in SIM mode. The validity of this approach is demonstrated by examination of the extraction efficiency, selectivity, sensitivity, and assay precision and accuracy.

3.1. Mass spectrometry

Background subtracted mass spectra of the TMS ethers of **I** and **II** are shown in Fig. 2A and B, respectively. The base peak at m/z 336 in both spectra is likely produced by a cleavage at the aliphatic nitrogen. This fragmentation pathway would lead to the formation of the m/z 336 ion for **I** by loss of a fluorobenzyl group and to the same ion for **II** by loss of a benzyl group. These fragments are also observed as charged species of m/z 123 in the spectrum of **I** and as m/z 105 in the spectrum of **II**.

3.2. Chromatography

Both **I** and **II**, even as TMS ethers, are relatively nonvolatile and chromatograph with difficulty. A column temperature of 350°C was required to elute both analytes in less than 10 min. Problems due to difficulties in vaporization or condensation in cooler portions of the GC–MS system may result in poor performance of the assay. It is essential that the sample extracts be completely dry prior to derivatization. Ethyl acetate and pyridine were added to the extracts to dissolve the analytes and absorb residual moisture, respectively, before adding BSTFA. Also, maintaining an excess of derivatization reagent in the injection solvent (1:1 ratio of BSTFA to tridecane) provided stability of a derivatized sample for at least 4 days.

Fig. 3A Fig. 4A demonstrate typical separations of the drug and internal standard by capillary GC–MS from dog and rat plasma, respectively. The profile observed in Fig. 3A was obtained from analysis of a dog plasma sample fortified with **I** (0.5 ng/ml) and **II** (4.6 ng/ml) and monitored at m/z

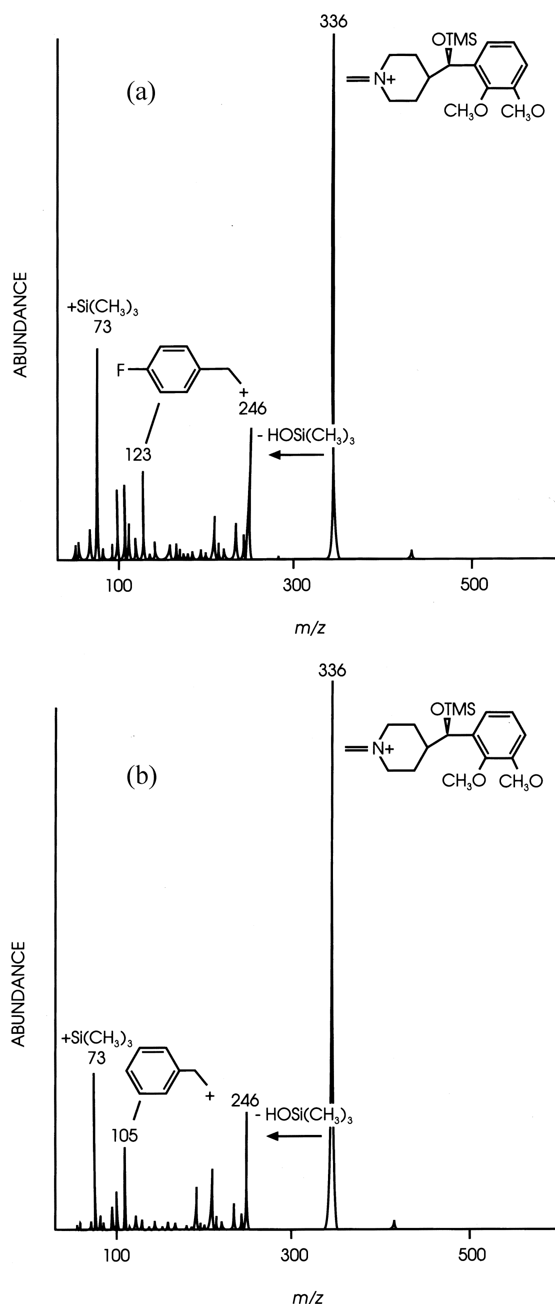

Fig. 2. EI mass spectra of the TMS ethers of **I** and **II**.

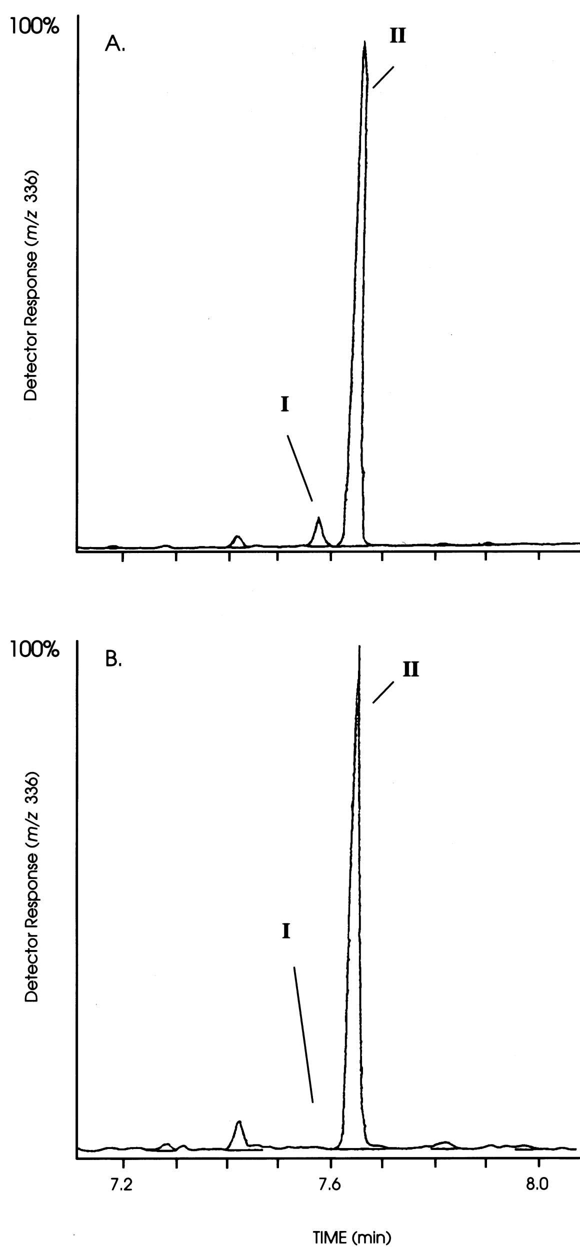

Fig. 3. Mass chromatograms (m/z 336) obtained by GC–MS analysis of a dog plasma quality control standard (panel A) that had been fortified with **I** (0.5 ng/ml) and **II** (4.6 ng/ml) or plasma containing only **II** (4.6 ng/ml, panel B) after extraction, derivatization and GC separation.

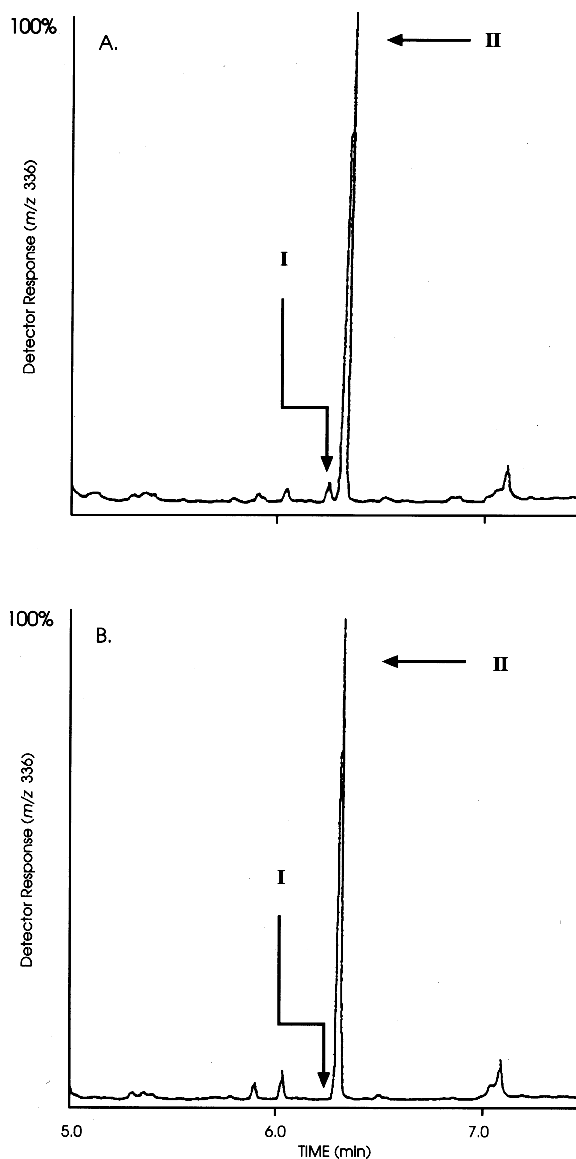

Fig. 4. Mass chromatograms (m/z 336) obtained by GC–MS analysis of a rat plasma quality control standard (panel A) that had been fortified with **I** (0.5 ng/ml) and **II** (4.6 ng/ml) or plasma containing only **II** (4.6 ng/ml, panel B) after extraction, derivatization and GC separation.

336. Baseline separation is obtained for the TMS ethers, with retention times of 7.57 and 7.65 min for **I** and **II** respectively. Similar results were obtained from rat plasma samples fortified with drug and internal standard at the same concentrations (Fig. 4A). Retention times of **I** and **II** from rat plasma were 6.28 and 6.35 min respectively. The slight difference in retention times between dog and rat analyses is due to the fact that duplicate GC–MS

systems were used, one for the analysis of rat samples and one for dog samples. The inlet septum, inlet liner were changed and the ion source cleaned daily to maintain sufficient resolution and sensitivity. Optimal results were also obtained when the ion source was cleaned weekly. The capillary column was found to last for over 2000 injections.

3.3. Extraction efficiency

Due to the involved nature of the procedure, which required formation of a TMS ether derivative and two evaporations to dryness, and to the variability observed with the GC–MS response, a simple comparison of absolute peak area of extracted samples with those from nonextracted samples was not feasible. Instead, a matrix design comparing the response factors for **I** in extracted and nonextracted samples to the nonextracted internal standard was used. Likewise, the response factor for the internal standard from extracted and nonextracted samples was compared to that of M100907 from nonextracted samples.

Absolute recoveries of **I** were determined at concentrations of 0.5 ng/ml and 5.0 ng/ml. The mean recovery ($n=3$) from dog and rat plasma at 0.5 ng/ml was 81% and 101%, respectively. The mean recovery ($n=3$) at the higher concentration, 5 ng/ml, from dog and rat plasma was 85% and 89%, respectively. The mean absolute recoveries of **II** from dog and rat plasma were 84% and 89% ($n=6$), respectively.

3.4. Selectivity/specificity

During the development and validation of this method in dog and rat plasma, multiple lots of control plasma were examined for endogenous compounds that would interfere with the determination of **I** or **II**. No such interferences were encountered. Fig. 3B Fig. 4B demonstrate the lack of an interfering ion co-eluting with the TMS ether of **I**. Although not shown, there were no interfering ions co-eluting with the TMS ether of **II** in blank samples from either plasma matrix. Another indication of the specificity of the method was observed by the lack of interferences from pre-dose plasma samples from rat or dog given the drug or from animals given only the dosing vehicle. There was also no indication of interferences with the peaks corresponding to either **I** or **II** during the analysis of study samples from any of the pharmaco- or toxicokinetic studies. This is demonstrated in Fig. 5A and B. Fig. 5A presents the chromatographic results from a 24 h plasma sample

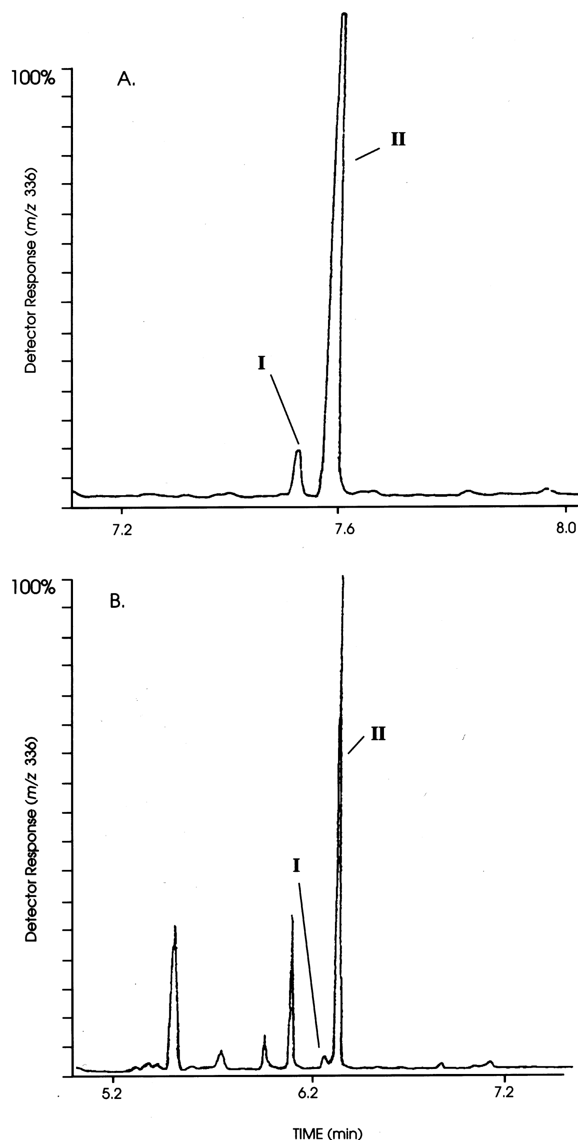

Fig. 5. Mass chromatograms (m/z 336) obtained by GC–MS analysis of a 24-h plasma sample (panel A) from a dog given an oral dose of **I** (6 mg/kg) and a 12-h plasma sample (panel B) from a rat given an oral dose of **I** (100 mg/kg) after extraction, derivatization and GC separation.

from a dog given **I** (6 mg/kg) orally. The measured concentration of **I** in this sample was 0.55 ng/ml. Fig. 5B presents the chromatographic results from a 12 h plasma sample from a rat given **I** (100 mg/kg) orally. The measured concentration of **I** in that sample was 0.32 ng/ml. Selected samples from both rat and dog treated with **I** were examined by full scan mass spectrometry and the spectra were congruent with an authentic standard, with no indication of an interference.

3.5. Accuracy and precision

The assay was validated by assaying five calibration standards (n =2) over the concentration range of 0.1 to 10 ng/ml and replicates of three quality control standards on three separate days. The line of best-fit was determined daily by using a weighted ($1/x$) quadratic least-squares regression analysis. The weighting factor of reciprocal concentration was used to ensure homoscedasticity over the concentration range [4]. The use of quadratic regression was dictated by the nonlinear response of the mass selective detector over the concentration range studied.

The correlation coefficient for the calibration curve over the validation and analysis period ranged from 0.989 to 0.999. The reproducibility of the curves is demonstrated in Table 1, which list the variation of the calibration standards to the regression line. The precision (% R.S.D.) ranged from 9.5 to 14.9% in dog plasma and from 1.7 to 13.4% in rat plasma. (Table 1). The corresponding ranges of accuracy (%R.E.) were −10.7 to 22.2% in dog plasma and −6.2 to 11.4% in rat plasma.

Assay precision and accuracy was determined by analyzing three replicate QC samples on two different days and six replicates on a third day. The results are summarized in Table 2. Within-day assay precision (%C.V.) for measuring concentrations of **I** ranged from 0.5 to 17% for dog plasma and from 1.7 to 18.5% for rat plasma. The corresponding within-day assay accuracy (% relative error) ranged from −11.6 to 17.3% in dog and −8.8 to 7.0% in rat. Between-day assay precision (%C.V.) for measuring concentrations of **I** ranged from 1.8 to 17.6% for dog plasma and from 1.6 to 15.1% for rat plasma. The corresponding between-day assay accuracy (% relative error) ranged from −6.0 to 12.0% in dog and −5.5 to 8.0% in rat.

The lower limit of quantitation was demonstrated in dog and rat calibration standards at 0.1 ng/ml (Table 1). It was also confirmed from three days of validation (n=11) and in rat from a single day of analysis (n=6) at a concentration of 0.15 ng/ml (Table 2). The %R.S.D. and %R.E. from either the 0.1 or 0.15 ng/ml concentration in either matrix were comparable with less than a ±20% deviation.

Table 1
Precision and accuracy of the assay for **I** in dog and rat plasma calibration standards

Concentration added (ng/ml)	n	Concentration found (ng/ml)	Relative standard deviation (%R.S.D.)	Relative error (%R.E.)
Dog plasma				
0.1	6	0.12	12.8	22.2
0.25	5	0.24	14.9	−6.0
0.5	6	0.45	11.5	−10.7
1	6	0.93	11.7	−7.3
10	6	11.02	9.5	10.2
Rat plasma				
0.1	4	0.11	13.4	11.4
0.25	6	0.25	9.0	1.7
0.5	6	0.48	7.9	−3.3
1	6	0.94	2.8	−6.2
10	6	9.98	1.7	−0.2

Table 2
Precision and accuracy of the assay for **I** in dog and rat plasma quality control standards

Concentration added (ng/ml)	Within-day				Between-day			
	n	Concentration found (ng/ml)	R.S.D.[a] (%)	Relative error (%)	*N*	Concentration found (ng/ml)	R.S.D. (%)	Relative error (%)
Dog plasma								
0.15	6	0.18	17.0	17.3	11	0.17	17.6	12.0
0.5	6	0.44	7.2	−11.6	13	0.47	14.2	−6.0
2.5	6	2.69	3.3	7.6	12	2.77	5.5	10.8
7.5	5	7.76	0.5	3.5	10	7.87	1.8	4.9
Rat plasma								
0.15	6	0.11	12.5	−8.8	–[b]	–	–	–
0.5	6	0.49	18.5	−3.0	12	0.47	15.1	−5.5
2.5	6	2.67	1.7	7.0	12	2.70	3.1	8.0
7.5	6	7.27	1.9	−3.3	12	7.29	1.6	−2.8

[a] Relative standard deviation.
[b] Not determined.

These results proved acceptable accuracy and precision at these low concentrations.

3.6. Stability

Periodic analysis of the stock solution demonstrated the lack of a stability issue in that solvent.

The stability of the analytes in the injection solvent was demonstrated in dog plasma samples after 4 days at room temperature and from rat plasma samples after 5 days at room temperature. Repeat analysis of the same samples on day 0 and day 4/5 resulted in differences in the response factors of no greater than 10% (Table 3). The effect of three freeze–thaw cycles on the stability of the compound was also evaluated by determining the recovery of the drug at two concentrations (0.5 and 7.5 ng/ml). The average recovery in dog plasma ($N=6$) was 87.1% and in rat plasma ($N=6$) 102.5% (Table 3).

The plasma stability of **I** when stored at −20°C was examined at two concentrations in dog and rat plasma (0.5 and 7.5 ng/ml) from the same quality control pools used in the three days of validation. The mean observed concentrations of **I** in dog plasma stored up to 46 days where within ±10% of the nominal value with a mean recovery of 90.4% (Table 3). Concentrations of **I** in rat plasma after 77 days of freezer storage where within ±10% of the nominal value and the mean recovery was 104.0% (Table 3).

Table 3
Stability of **I** in different solvents

Matrix	Temperature	Days	Dog (*n*)	Recovery (%)	Days	Rat (*n*)	Recovery (%)
Injections Solvent	Room temperature	4	12	90.4	5	8	101.7
Plasma	Three freeze–thaw cycles	3	6	87.1	3	6	102.5
Plasma	−20°C	46	6	90.4	77	6	104.0

4. Conclusion

Sample purification by C_1 SPE followed by derivatization with BSTFA, separation by capillary GC, and detection by MSD in SIM mode allowed for quantification of **I** in both rat and dog plasma over the concentration range of 0.1 to 10 ng/ml. The method is both specific and sensitive down to the picomolar level in plasma. The precision and accuracy of the method are adequate for use in toxicokinetic and pharmacokinetic studies. The compound appears to be stable in either rat or dog plasma when stored frozen at −20°C.

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