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A highly sensitive LC-MS/MS method for the determination of *S*-citalopram in rat plasma: application to a pharmacokinetic study in rats

P. S. Suresh[a], Sanjeev Giri[a], Raghib Husain[a] and Ramesh Mullangi[b]*

ABSTRACT: A highly sensitive, rapid assay method has been developed and validated for the estimation of *S*-citalopram (*S*-CPM) in rat plasma with liquid chromatography coupled to tandem mass spectrometry with electrospray ionization in the positive-ion mode. The assay procedure involves a simple liquid–liquid extraction of *S*-CPM and phenacetin (internal standard, IS) from rat plasma with *t*-butyl methyl ether. Chromatographic separation was operated with 0.2% formic acid:acetonitrile (20:80, v/v) at a flow rate of 0.50 mL/min on a Symmetry Shield RP_{18} column with a total run time of 3.0 min. The MS/MS ion transitions monitored were 325.26 → 109.10 for *S*-CPM and 180.10 → 110.10 for IS. Method validation and pre-clinical sample analysis were performed as per FDA guidelines and the results met the acceptance criteria. The lower limit of quantitation achieved was 0.5 ng/mL and the linearity was observed from 0.5 to 5000 ng/mL. The intra- and inter-day precisions were in the range of 1.14–5.56 and 0.25–12.3%, respectively. This novel method has been applied to a pharmacokinetic study and to estimate brain-to-plasma ratio of *S*-CPM in rats. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: *S*-Citalopram; LC-MS/MS; method validation; rat plasma; pharmacokinetics

Introduction

(+)-*S*-Citalopram (*S*-CPM; CAS no. 128196–01–0; Fig. 1), chemically *S*-(+)- 1-[3-(dimethylamino)propyl]-1-(*p*-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile belongs to a class of drugs known as selective serotonin reuptake inhibitors (SSRI). Lexapro® (*S*-citalopram oxalate) is commercially available as film-coated tablets (5, 10 and 20 mg) and oral solution. *S*-CPM is used in the treatment of depression and anxiety. *In vitro* and *in vivo* studies in animals suggest that *S*-CPM is a highly selective SSRI with minimal effects on norepinephrine and dopamine neuronal reuptake. *S*-CPM is twice as potent as the reacemic mixture as a 5-HT reuptake inhibitor and 100 times more that than its antipode, i.e. (–)-*R*-citalopram. Following oral single and multiple dosing in humans *S*-CPM has shown linear dose proportional pharmacokinetics in the dose range of 10–30 mg/day. Following a single oral dose (tablet or solution, 20 mg) of *S*-CPM to humans, the peak plasma concentrations (C_{max}) were achieved at about 5 h (T_{max}). Absorption of *S*-CPM was not affected by food. Biotransformation of *S*-CPM occurs mainly in liver and converts into demthyl- and didemthyl-*S*-CPM. *In vitro* studies using human liver microsomes indicated that cytochrome P450 3A4 and cytochrome P450 2C19 are the primary isozymes involved in the *N*-demethylation of *S*-CPM. Its protein binding was found to be ≈56%. The mean terminal half-life ($t_{½}$) is around 27–32 h. Nausea, somnolence and gastro-intestinal side effects are common side effects with *S*-CPM.

Few LC-MS (Pistos *et al*., 2004; Singh *et al*., 2004) or LC-MS/MS methods have been reported for estimation of citalopram (CPM) (Sauvage *et al*., 2006; Rocha *et al*., 2007; de Castro *et al*., 2008) or *S*-CPM (Singh *et al*., 2004) in biological matrices. The LC-MS method developed and validated by Pistos *et al*. (2004) has a longer run time (10 min) with an lower limit of quantitation (LLOQ) of 0.5 ng/mL. Subsequently Singh *et al*. (2004) developed an LC-MS method with an LLOQ of 1 ng/mL. Sauvage *et al*. (2006) reported a fully automated turbulent flow LC-MS/MS method for the estimation of CPM along with 12 other antidepressants with an LLOQ of 10 ng/mL using 100 µL of human serum. Rocha *et al*. (2007) developed and validated an enantioselective LC-MS/MS for the estimation of CPM and its metabolite (desmethyl CPM) in rat and human plasma (1 mL) with an LLOQ of 0.1 ng/mL. Recently, de Castro *et al*. (2008) developed a simultaneous estimation of CPM along with eight other antidepressants in human saliva and plasma with an LLOQ of 2 ng/mL using 200 µL of plasma/saliva. Although the reported LC-MS/MS methods are sensitive enough, the reported LLOQ amongst them was 0.1 ng/mL using 1 mL plasma volume (Rocha *et al*., 2007). In the present paper, we present an LC-MS/MS method with an LLOQ of 0.5 ng/mL using a

* Correspondence to: R. Mullangi, Jubilant Innovation, 2nd Stage, Industrial Suburb, Yeshwanthpur, Bangalore-560 022, India. E-mail: mullangi_ramesh@jubilantinnovation.com

[a] Drug Metabolism and Pharmacokinetics, Jubilant Biosys Ltd, Industrial Suburb, Yeshwanthpur, Bangalore-560 022, India

[b] Jubilant Innovation, 2nd Stage, Industrial Suburb, Yeshwanthpur, Bangalore-560 022, India

Abbreviations used: CE, collision energy; DP, declustering potential; CXP, collision exit potential; EP, entrance potential; *S*-CPM, (+)-*S*-citalopram; SSRI, serotonin reuptake inhibitors.

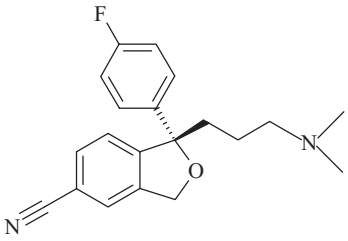

Figure 1. Structural representation of *S*-citalopram (*S*-CPM).

very low volume (50 µL) of rat plasma. The LLOQ achieved is 4-fold lower than the lowest reported LLOQ (Rocha *et al*., 2007) for CPM. Further the validated method was extended to estimate the *S*-CPM in rat brain homogenate. Our method involves simple liquid–liquid extraction sample processing (amicable for both plasma and brain homogenate) and has a run time of 3.0 min; hence our method gives higher throughput. As *S*-CPM exerts its pharmacological activity by inhibiting the reuptake of 5-HT in brain, we felt that it is worthwhile to assess the concentrations of *S*-CPM in brain and it will help to use plasma as a surrogate for the estimation of *S*-CPM in brain. The newly developed LC-MS/MS method was successfully used in a rat pharmacokinetic study and to assess the brain-to-plasma concentration of *S*-CPM following administration of a 10 mg/kg oral dose.

Experimental

Chemicals and Reagents

S-CPM and phenacetin (IS) were procured from Jubilant Organosys (Noida, New Delhi, India). HPLC-grade acetonitrile and methanol were purchased from Rankem (Ranbaxy Fine Chemicals Limited, New Delhi, India). Analytical grade formic acid was purchased from S.D. Fine Chemicals (Mumbai, India). Tris–HCl buffer was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Sprague–Dawley male rats were purchased from Reliance Life Sciences (Mumbai, India).

HPLC Operating Conditions

A Shimadzu VP (Shimadzu, Japan) LC system equipped with degasser (G1379A), quaternary pump (10ADvp), column oven (CTO-10ASvp) and auto-sampler (SIL-HTC) along with a system controller (SCL-10Avp) was used to inject 2 µL aliquots of the processed samples on a Symmetry Shield RP_{18} column (50 × 4.6 mm, 3.5 µm, Waters Corporation, Ireland, UK), which was kept at ambient temperature (24 ± 2°C). The isocratic mobile phase, a mixture of 0.2% formic acid and acetonitrile mixture (20:80, v/v) was filtered through a 0.45 µm membrane filter (XI5522050) (Millipore, USA or equivalent) and then degassed ultrasonically for 5 min was delivered at a flow rate of 0.50 mL/min into the mass spectrometer electro spray ionization chamber.

Mass Spectrometry Operating Conditions

Quantitation was achieved by MS/MS detection in positive ion mode for analyte and IS using a MDS Sciex (Foster City, CA, USA) API 4000 mass spectrometer, equipped with a Turboionspray™ interface at 500°C. The common parameters, i.e. curtain gas, nebulizer gas, auxillary gas and collision gas, were set at 10, 35, 40 and 6 psi, respectively. The compounds parameters, i.e. declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP) for *S*-CPM and IS were 81, 43, 6, 10 V and 34, 30, 8, 9 V, respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the *m/z* 325.26 precursor ion to the *m/z* 109.10 product ion for *S*-CPM and *m/z* 180.10 precursor ion to the *m/z* 110.10 product ion for IS. Quadrupole Q1 was set on low resolution where as Q3 was set on unit resolution. The analytical data were processed by Analyst software (version 1.4.2).

Preparation of Stock and Standard Solutions

Primary stock solutions of *S*-CPM for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solutions were prepared in methanol (1000 µg/mL). The IS stock solution of 1000 µg/mL was prepared in methanol. The stock solutions of *S*-CPM and IS were stored at 4 °C, which were found to be stable for one month (data not shown) and successively diluted with methanol to prepare working solutions to prepare the calibration curve (CC). Another set of working stock solutions of *S*-CPM were made in methanol (from primary stock) for preparation of QC samples. Working stock solutions were stored at approximately 4°C for a week (data not shown). Appropriate dilutions of *S*-CPM stock solution were made in methanol to produce working stock solutions of 0.50, 1.00, 10, 50, 100, 500, 750 and 1000 ng/mL. Working stocks were used to prepare plasma calibration standards. A working IS solution (200 ng/mL) was prepared in methanol. Calibration samples were prepared by spiking 45 µL of control rat plasma with the appropriate working solution of the analyte (5 µL) and IS (10 µL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking control rat plasma in bulk with *S*-CPM at appropriate concentrations (0.50, 1.50, 500 and 800 ng/mL) and 50 µL plasma aliquots were distributed into different tubes. All the samples were stored at –80 ± 10°C.

Recovery

The efficiency of *S*-CPM and IS extraction from rat plasma was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 6$) with the response of analytes from post extracted plasma standard sample at equivalent concentrations (Dams *et al*., 2003) by liquid–liquid extraction. Recoveries of *S*-CPM were determined at QC low and QC high concentrations, i.e. 1.50 and 800 ng/mL, whereas the recovery of the IS was determined at a single concentration of 200 ng/mL.

Sample Preparation

A simple liquid–liquid extraction method was followed for extraction of *S*-CPM from rat plasma. To an aliquot of 50 µL plasma, IS solution (10 µL of 200 ng/mL) was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 2 mL of *ter*-butyl methyl ether (TBME), the mixture was vortexed for 2 min, followed by centrifugation for 10 min at 3200 rpm on Multifuge 3_{SR} (Heraus, Germany). The organic layer (1.8 mL) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Turbovap®, Zymark® Kopkinton, MA, USA). The residue was reconstituted in 200 µL of the mobile phase and 2 µL was injected onto LC-MS/MS system.

Validation Procedures

A full validation according to the FDA guidelines (US DHHS, FDA, CDER, 2001) was performed for the assay in rat plasma.

Specificity and selectivity. The specificity of the method was evaluated by analyzing rat plasma samples from at least six different lots to investigate the potential interferences at the LC peak region for analyte and IS.

Matrix effect. The effect of rat plasma constituents over the ionization of *S*-CPM and IS was determined by comparing the responses of the post extracted plasma QC samples ($n = 6$) with the response of analytes from neat standard samples (5 µL of required working stock sample spiked into 45 µL of methanol instead of blank plasma) at equivalent concentrations (Hubert *et al*., 1999; Dams *et al*., 2003). Matrix effect was determined at low and high concentrations, i.e. 1.50 and 800 ng/mL, whereas the matrix effect over the IS was determined at a single concentration of 200 ng/mL.

Calibration curve. The eight point calibration curve (0.50, 1.00, 10, 50, 100, 500, 750 and 1000 ng/mL) was constructed by plotting the peak area ratio of *S*-CPM:IS against the nominal concentration of calibration standards in rat plasma. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of $1/X^2$ (X = concentration) weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20% (US DHHS, FDA, CDER, 2001).

Precision and accuracy. The intra-assay precision and accuracy were estimated by analyzing six replicates containing *S*-CPM at four different QC levels: 0.50, 1.50, 500 and 800 ng/mL in plasma. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within ± 15% deviation (SD) from the nominal values and a precision of within ± 15% relative standard deviation (RSD) except for LLOQ, where it should not exceed ± 20% (US DHHS, FDA, CDER, 2001).

Stability experiments. The stability of *S*-CPM and IS in the injection solvent was determined periodically by injecting replicate preparations of processed plasma samples for up to 12 h (in the autosampler at 4°C) after the initial injection. The peak-areas of the analyte and IS obtained in the initial cycle were used as the reference to determine the stability at subsequent points. Stability of *S*-CPM in plasma during 6 h (bench-top) was determined at ambient temperature (24 ± 2°C) at two concentrations (1.50 and 800 ng/mL) in six replicates. Freezer stability of *S*-CPM in rat plasma/brain was assessed by analyzing the LQC and HQC samples stored at –80 ± 10°C for at least 30 days. The stability of *S*-CPM in rat plasma following three freeze–thaw cycles was assessed using QC samples spiked with *S*-CPM. The samples were stored at –80 ± 10°C between freeze–thaw cycles. The samples were thawed by allowing them to stand (unassisted) at room temperature for approximately 2 h. The samples were then returned to the freezer. The samples were processed using the same procedure as described in the Sample Preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD).

Dilution effect. Dilution effect was investigated to ensure that samples could be diluted with blank plasma without affecting the final concentration. *S*-CPM spiked rat plasma samples prepared at two concentrations (50 and 9000 ng/mL) of *S*-CPM were diluted with pooled rat plasma at dilution factors of 5 and 10 in six replicates and analyzed. The six replicates should have precision of ≤15% and accuracy of 100 ± 15%.

In vivo Studies in Rats

A pharmacokinetic (PK) study was performed in over night (~12 h) fasted healthy male Sprague–Dawley rats ($n = 4$, weight range 210–220 g) following approval from the ethical committee. During fasting time animals had free access to water. Blood samples were obtained following oral administration of 10 mg/kg *S*-CPM (in the form of a suspension, prepared using 20 µL of Tween-80 + 0.5% methyl cellulose) into polypropylene tubes containing EDTA solution as an anti-coagulant at pre-dose, 0.25, 0.5, 1, 2, 4, 6, 10 and 24 h. During the PK study another set of overnight (~12 h) fasted animals ($n = 6$, weight range 212–225 g) were also dosed with 10 mg/kg of *S*-CPM suspension and the brain tissue was collected at 1, 4 and 8 h (each time point two animals were sacrificed to collect the brain tissue along with blood). In both the experiments animals were allowed to eat feed 3 h post-dose of *S*-CPM. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760***g*** for 5 min and stored frozen at –80 ± 10°C until analysis. Following the collection of brain tissue in a 15 mL round-bottom screw-capped vial, Tris–HCl buffer was added and homogenated with a homogenizer (Miccra D-9) and stored frozen at –80 ± 10°C until analysis. Plasma (50 µL) or brain homogenate (50 µL) samples were spiked with IS and processed as described above. Along with PK samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among calibrators and unknown samples in the analytical run; not more than 33% of the QC samples were greater than ± 15% of the nominal concentration. Plasma concentration–time data of CPM was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA).

Results

Liquid Chromatography

The feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid along with altered flow rates (in the range of 0.1–0.5 mL/min) was tested for complete chromatographic resolution of *S*-CPM and IS (data not shown). The resolution of peaks was achieved with 0.2% formic acid:acetonitrile (20:80, v/v) with a flow rate of 0.5 mL/min, on a Symmetry Shield RP_{18} column (50 × 4.6 mm, 3.5 µm, Waters, UK) and was found to be suitable for the determination of electrospray response for *S*-CPM and IS.

Mass Spectroscopy

In order to optimize ESI conditions for *S*-CPM and IS, quadrupole full scans were carried out in positive ion detection mode. During

a direct infusion experiment, the mass spectra for *S*-CPM and IS revealed peaks at *m/z* 325.26 and 180.10, respectively, as protonated molecular ions, $[M + H]^+$. Following detailed optimization of mass spectrometry conditions (provided in the Instrumentation and Chromatographic Conditions section) the *m/z* 325.26 precursor ion to the *m/z* 109.10 was used for quantification of *S*-CPM. Similarly, for IS the *m/z* 180.10 precursor ion to the *m/z* 110.10 was used for quantification purpose.

Recovery

A simple liquid–liquid extraction with TBME following alkalization proved to be robust and provided the cleanest samples. The results of the comparison of neat standards vs plasma-extracted standards were estimated for *S*-CPM at 1.50 and 800 ng/mL and the mean recovery was found to be 61.28 ± 5.58 and 52.35 ± 2.44%, respectively. The recovery of IS at 200 ng/mL was 85.47 ± 6.92%.

Matrix Effect, Specificity and Selectivity

Average matrix factor values (matrix factor = response of post spiked concentrations/response of neat concentrations) obtained were +0.60 (CV: 3.21%, $n = 6$) and +0.71 (CV: 12.70%, $n = 6$) for *S*-CPM in rat plasma at QC low (1.50 ng/mL) and QC high (800 ng/mL) concentrations, respectively. No significant peak area differences were observed. The matrix effect on IS was found to be +1.16 (CV: 14.95%, $n = 6$) at the tested concentration of 200 ng/mL. Overall it was found that the plasma extract has a small impact on the ionization of analyte and IS.

Figure 2 shows a typical chromatogram for the control rat plasma (free of analyte and IS), control rat plasma spiked with IS, rat plasma spiked with *S*-CPM at LLOQ and IS and an *in vivo* plasma sample obtained at 1 h after oral administration of *S*-CPM. No interfering peaks from endogenous compounds are observed at the retention times of analyte and IS in the matrix. The retention times of *S*-CPM and IS were ~0.66 and 1.40 min, respectively. The total chromatographic run time was 3.0 min.

Calibration Curve

The plasma homogenate calibration curve was constructed using eight calibration standards (0.5–1000 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to the $y = mx + c$ using a weighing factor ($1/X^2$). The average regression ($n = 4$) was found to be ≥0.995. The lowest concentration with the RSD < 20% was taken as LLOQ and was found to be 0.5 ng/mL. The percentage accuracy observed for the mean of back-calculated concentrations for four calibration curves for *S*-CPM was within 88.4–109.8, while the precision (%CV) values ranged from 0.47 to 9.08.

Accuracy and Precision

Accuracy and precision data for intra- and inter-day plasma/brain homogenate samples are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

Stability

The predicted concentrations for *S*-CPM at 1.50 and 800 ng/mL samples deviated within ±15% of the nominal concentrations in a battery of stability tests: in-injector (12 h), bench-top (6 h), repeated three freeze–thaw cycles and freezer stability at −80 ± 10°C for at least for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

Dilution Effect

The standard curve can be extended up to 9000 ng/mL without affecting the final concentrations. The results have shown that the precision and accuracy for six replicates of diluted samples were within the acceptance range (data not shown).

In vivo Studies

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of *S*-CPM in rats. Profiles of the mean plasma concentration vs time were shown in Fig. 3. Maximum concentration in plasma (C_{max} 417 ± 65.8 ng/mL) was achieved at 0.67 ± 0.29 h (T_{max}). The half-life ($t_{1/2}$) of *S*-CPM was 2.00 ± 0.25 h, while the $AUC_{(0-\infty)}$ was 1375 ± 131 ng h/mL. The higher sensitivity of this method compared with the current existing methods in literature facilitates the quantitation of *S*-CPM at lower concentrations with high turnover.

Although we have not performed full validation of *S*-CPM in brain homogenate, the recovery of *S*-CPM from brain homogenate at 1.50 and 800 ng/mL was found to be 63.82 ± 4.62 and 57.35 ± 4.26%, respectively, using the similar extraction process used for plasma. The percentage accuracy observed for the mean of back-calculated concentrations for two calibration curves for *S*-CPM in brain homogenate was within acceptable limits (data not shown). Hence we have used the plasma calibration curve to quantitate the rat brain concentrations following oral dosing of *S*-CPM to rats. The QCs spiked in brain homogenate met the acceptance criteria (data not shown) when the concentrations were back-calculated using plasma linearity. Further, there was no interference from the *S*-CPM free brain homogenate (collected from four rats) at the retention times of analyte and IS. Figure 4 shows the brain to plasma ratio at 1, 4 and 8 h time points. From this figure it is evident that around the C_{max} time point the brain concentrations of *S*-CPM were higher (~3 fold) than plasma concentrations. The brain-to-plasma ratio profile established by us will help the scientists to consider plasma as a surrogate to speculate the brain concentrations.

Conclusion

A method using LC-ESI-MS/MS for the determination of *S*-CPM in rat plasma and brain homogenate employing simple liquid–liquid extraction was developed. The method is rapid, simple, specific and sensitive, and additionally demonstrates good accuracy and precision. Compared with the published methods, the present method features high selectivity and sensitivity with an LLOQ of 0.5 ng/mL. We believe that this high-throughput method could provide a useful tool for the determination of *S*-CPM in plasma and brain homogenate. The established method was successfully applied to a rat pharmacokinetic study and to assess the brain-to-plasma ratio.

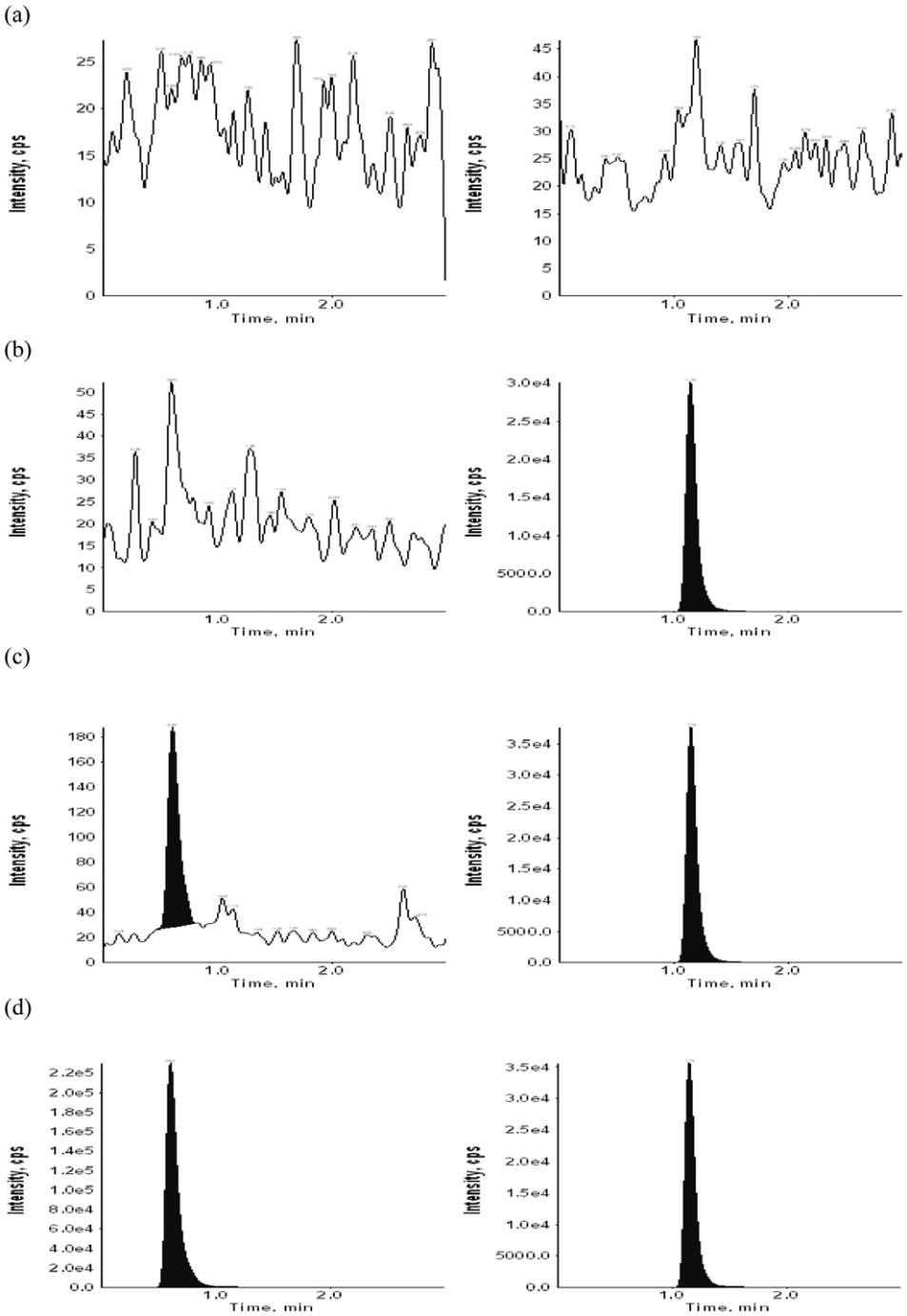

Figure 2. Typical MRM chromatograms of *S*-CPM (left panel) and IS (right panel) in (a) rat blank plasma, (b) rat plasma spiked with IS, (c) rat plasma spiked with *S*-CPM at LLOQ (0.50 ng/mL) and (d) a 1 h plasma sample showing *S*-CPM (413.61 ng/mL) peak obtained following 10 mg/kg oral dose of *S*-CPM to rats.

Table 1. Intra- and inter-day precision of determination of S-CPM in rat plasma

Theoretical concentration (ng/mL)	Run	Measured concentration (ng/mL) Mean	SD	RSD	Accuracy (%)
Intra-day variation (six replicates at each concentration)					
0.50	1	0.60	0.01	1.17	113
	2	0.51	0.06	12.3	100
	3	0.48	0.04	7.29	93.3
	4	0.50	0.02	4.20	98.3
1.50	1	1.45	0.07	4.88	94.0
	2	1.45	0.03	1.95	94.3
	3	1.45	0.08	5.35	87.9
	4	1.61	0.08	4.82	104
500	1	503	5.82	1.16	98.3
	2	522	9.34	1.79	101
	3	518	4.41	0.85	101
	4	519	12.1	2.33	101
800	1	845	12.5	1.48	105
	2	793	1.97	0.25	99.2
	3	802	23.1	2.88	100
	4	877	8.03	0.92	109
Inter-day variation (Twenty four replicates at each concentration)					
0.50		0.54	0.03	5.56	100
1.50		1.50	0.08	5.33	97.6
500		522	10.5	2.01	101
800		837	9.53	1.14	104

RSD, Relative standard deviation (SD × 100/mean).

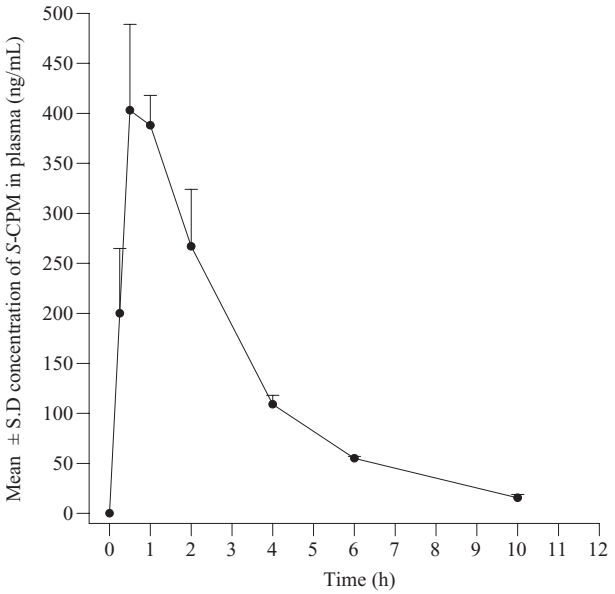

Figure 3. Mean ± SD plasma concentration–time profile of *S*-CPM in rat plasma following oral dosing of *S*-CPM to rats.

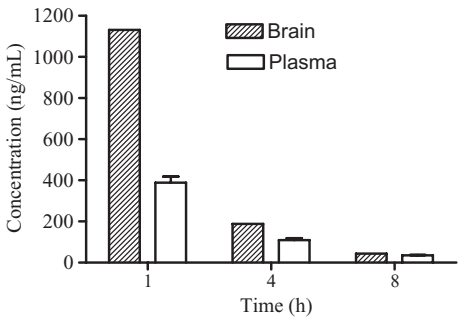

Figure 4. Brain-to-plasma ratio of *S*-CPM at 1, 4 and 8 h following 10 mg/kg oral dose of *S*-CPM to rats.

Table 2. Stability data *S*-CPM quality controls in rat plasma

Nominal concentration (ng/mL)	Stability	Mean ± SD[a], *n* = 6 (ng/mL)	Accuracy (%)[b]	Precision (%CV)
1.50	0 h (for all)	1.50 ± 0.05	97.5	3.33
	Third freeze–thaw	1.70 ± 0.05	110	3.26
	6 h (bench-top)	1.43 ± 0.08	92.9	5.97
	12 h (in-injector)	1.34 ± 0.01	87.5	1.40
	30 days at –80°C	1.58 ± 0.05	103	3.53
800	0 h (for all)	823 ± 35.7	103	4.33
	Third freeze–thaw	753 ± 25.4	94.1	3.37
	6 h (bench-top)	813 ± 62.9	101	7.73
	12 h (in-injector)	838 ± 20.1	104	2.40
	30 days at –80°C	784 ± 31.9	98.1	4.07

[a] Back-calculated plasma concentrations. [b] (Mean assayed concentration/mean assayed concentration at 0 h) × 100.

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