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Measurement of NMDA Receptor Antagonist, CPP, in Mouse Plasma and Brain Tissue Following Systematic Administration Using Ion-Pair LCMS/MS

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Abstract

(RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) is a competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor and is routinely used with rodent models to investigate the role of NMDA receptors in brain function. This highly polar compound is difficult to separate from biological matrices. A reliable and sensitive assay was developed for the determination of CPP in plasma and tissue. In order to overcome the challenges relating to the physicochemical properties of CPP we employed an initial separation using solid phase extraction harnessing mixed-mode anion exchange. Then an ion-pair UPLC C18 separation was performed followed by MS/MS with a Waters Acquity UPLC interfaced to an AB Sciex QTrap 5500 mass spectrometer, which was operated in positive ion ESI mode. Multiple reaction monitoring (MRM) mode was utilized to detect the analyte and internal standard. The precursor to product ions used for quantitation for CPP and internal standard were m/z 252.958 $\rightarrow$ 207.100 and 334.955 $\rightarrow$ 136.033, respectively. This method was applied to a pharmacokinetic study and examined brain tissue and plasma concentrations following intravenous and intraperitoneal injections of CPP. The elimination half-life ($t_{1/2}$) of CPP was 8.8 minutes in plasma and 14.3 minutes in brain tissue, and the plasma to brain concentration ratio was about 18:1. This pharmacokinetic data will aid the interpretation of the vast number of studies using CPP to investigate NMDA receptor function in rodents and the method itself can be used to study many other highly polar analytes of interest.

Introduction

The purpose of our work was to determine the concentrations of (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), a competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor, in plasma and brain tissue following systemic

administration in rodent models. Determining tissue-specific drug concentrations that correspond to specific behavioural endpoints will guide the design and interpretation of studies utilizing CPP for companion experiments performed *in vivo* and *in vitro*.

The NMDA receptor is a glutamate-activated ion channel that is permeable to cations, including sodium and calcium. It contributes to excitatory synaptic transmission throughout the central nervous system, and is essential to higher cognitive functions [1]. The role of NMDA receptors in learning and memory has been extensively documented [2-6]. Many stimulus paradigms that induce long-term potentiation, a form of synaptic plasticity that is widely studied as a cellular correlate of memory, depend critically on activation of NMDA receptors to trigger downstream signalling processes [7-11]. The role of NMDA receptors goes beyond memory, as these receptors influence a vast number of cognitive processes and neurological diseases related to excitatory synaptic transmission [12]. NMDA receptor modulation has been shown to influence Alzheimer's disease [13-15], Parkinson's disease [16,17], general anaesthesia [18,19], depression [20,21], and neuropathic pain [22].

Research in all of these areas has utilized CPP as a pharmacological tool to investigate the role of NMDA receptors and the functional consequences of their impairment. CPP antagonizes the NMDA receptor by reversibly binding to the glutamate binding site [8]. CPP was synthesized as an analogue of 2-amino-5-phosphonopentanoic acid (AP5) and 2-amino-7-phosphonoheptanoic acid (AP7) [23], which are also commonly used to block NMDA receptors *in vitro*. CPP is highly selective for NMDA receptors [24], and is 5-fold more potent than AP5 or AP7. What makes CPP so useful experimentally is that, despite having hydrophilic properties, it crosses the blood-brain barrier [4, 24, 25]. Therefore, unlike most other competitive antagonists, it is effective even when administered systemically. CPP's high potency, specificity for NMDA receptors, and penetration of the blood-brain barrier has led to its wide spread adoption. At doses that range between 0.5 mg/kg to 10 mg/kg, systemic administration of CPP in rodents has been shown to suppress seizure activity [24,26], interfere with addiction paradigms [27], block stress-induced responses [28,29], produce antidepressant-like effects [30], disrupt neurogenesis [31], modulate Parkinson's disease models [32,33], increase amyloid β levels in Alzheimer's disease models [34], impair learning and memory [3-5,35], and block both long-term potentiation (LTP) and long-term depression (LTD) [36-38]. Although these studies have demonstrated the ability of CPP to alter a plethora of behavioral responses, the degree to which NMDA receptors in the CNS must be blocked in order to produce a given response remains unknown. The interpretation of *in vitro* studies that could provide this type of information will require detailed knowledge of the concentration of antagonist that reaches the brain following systemic administration.

Published pharmacokinetic analyses of CPP are limited. A review of the literature identified only two papers that examined CPP concentrations following systemic administration, one utilizing liquid scintillation spectrometry and the second based on HPLC-UV analysis of a CPP derivative. In exploration of CPP's clinical potential, levels of [H^3]CPP in serum and CSF were measured in pigs following intravenous injection [25]. Absolute concentrations were not measured, but the half-life of CPP (81 ± 10 minutes) and the CSF:serum ratio (0.28 ± 0.03) were reported. In a second study, plasma concentrations of CPP were measured

1-5 hours following oral administration to baboons [26]. Concentrations of CPP in plasma ranged from 0.6 to 0.8 µg/ml over this timeframe following an oral dose that produced a potent anti-convulsant effect (32 mg/kg). These measurements utilized a derivatization protocol, but had a detection limit of only 80 ng/ml. A major advantage of using mass spectrometry over either previously described method is that with mass spectrometry the analyte can be identified both by its retention time and molecular weight. Using a triple quadrupole mass spectrometer, analytes can be fragmented and specific fragments can be monitored which eliminates most interference from complex biological matrices and allows for confident analyte detection and quantitation. With an interest in measuring CPP concentrations in plasma and brain tissue in rodents following systemic administration of relevant doses, the present work aimed to develop a reliable and reproducible analytical method.

Assay development was challenging because of the physicochemical properties of CPP (**Figure 1A**). Being a highly polar compound and lacking a chromophore that would allow routine UV-Vis spectrophotometric analysis, CPP required suitable methodologies to be optimized for extraction from tissue or plasma, sample preparation, and HPLC separation prior to mass spectrometry analysis. Unfortunately, CPP would not elute from HILIC columns, which makes it difficult to achieve adequate elution or separation. Additionally, CPP is poorly retained on C18 columns under standard conditions. To overcome these challenges we used an ion-pairing agent, HFBA, with a C18 column. In this study, we report a high quality method that allowed sensitive detection and accurate quantification of CPP in complex biological matrices.

Materials and Methods

Chemicals and Reagents

CPP was purchased from Tocris Bioscience (Bristol, UK). Internal standard (IS) isotopically labeled cyclic adenosine monophosphate ($^{13}\text{C}_5$ cAMP) was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Ammonium hydroxide solution and formic acid (mass-spec grade) were purchased from Sigma Aldrich (Saint Louis, MO). Heptafluorobutyric acid (HFBA) was purchased from Thermo Scientific Pierce (Rockford, IL). HPLC grade water, methanol, and acetonitrile (AcN), and hydrochloric acid (ACS reagent grade) were purchased from Fisher Scientific (Waltham, MA).

Calibration Standards/Quality Control Samples

A 10 mg/ml stock solution of CPP in water was prepared by dissolving 10 mg CPP powder in 1 mL of water and stored in a polypropylene centrifuge tube at -20°C . A 10 µg/ml stock solution of IS was prepared in water. Working solutions (10x) of CPP and IS were made by dilution in water and used to prepare calibration standard samples. Different calibration ranges were assessed for mouse plasma and brain tissue. For plasma six non-zero CPP standards, ranging from 5 ng/ml to 1500 ng/ml, were prepared by adding 10 µl of working solution of CPP and 10 µl IS working solution (0.5 µg/ml) to aliquots (100 µl) of plasma. For brain tissue five non-zero CPP standards, ranging from 0.9 ng/g to 44 ng/g, were prepared by adding 10 µl of working solution of CPP and 10 µl IS working solution (0.5 µg/ml) to

individual blank brain samples (0.34 g) in 2 ml 0.01N HCl prior to homogenization. Five replicates of all standards were prepared. Blank samples consisted of the appropriate matrix devoid of CPP or IS. Zero samples were prepared by adding 10 μ l of IS to plasma or brain blanks. Calibration curves were generated from the areas of the analyte and IS using a 1/x weighted linear regression for brain tissue samples and a 1/x weighted quadratic regression for plasma samples.

Sample Preparation

All experiments conformed to the guidelines laid out by the Guide for the Care and Use of Laboratory Animals, and were conducted with the approval of the University of Wisconsin-Madison (Madison, Wisconsin) Animal Care and Use Committee.

Trunk blood was collected in heparinized tubes following decapitation of anesthetized animals, and plasma was separated by centrifugation. Plasma samples were immediately processed after collection. Aliquots (100 μ l) of plasma samples were added to 10 μ l IS working solution (0.5 μ g/ml) in a 1.5 ml polypropylene centrifuge tube. To this 200 μ l 0.01N HCl was added, then samples were vortex-mixed and centrifuged at 14,000xg for 5 minutes at 4°C. The supernatant was added to 700 μ l 1.5% NH₄OH (v/v) in order to basify the solution prior to solid phase extraction (SPE).

Brain tissue was weighed immediately following decapitation and placed on ice. Tissue was then homogenized in glass test tubes with 2 ml 0.01N HCl using a rotor-stator homogenizer. The brain homogenate was transferred to 15 ml centrifuge tubes and centrifuged at 15,000xg for 25 minutes at 4°C. The supernatant was transferred to a fresh 15 ml conical polypropylene tube. The pelleted tissue was resuspended in 2 mL 0.01N HCl and centrifuged for a second time at 15,000xg for 25 minutes at 4°C. The supernatant from the second spin was combined with the supernatant from the first spin. 3.5 mL 5% NH₄OH (v/v) was then added in order to basify the solution prior to SPE.

SPE was performed using Oasis MAX SPE cartridges from Waters (Milford, MA). The SPE cartridge was conditioned with methanol, equilibrated with water, and then the basified sample was loaded. The cartridge was washed with 5% NH₄OH and then washed with methanol. Analyte was eluted with 8% formic acid/77% methanol (v/v).

Following SPE, samples were dried down in a speed vacuum concentrator. Plasma samples were resuspended in 60 μ L diluent (20 mM HFBA in 99:1 water:AcN) and brain tissue samples were resuspended in 20 μ L diluent. Samples were shaken, spun down with a centrifuge for 10 s, and transferred to HPLC vials.

Instrumentation

LC-MS/MS analysis was performed with a Waters Acquity binary pump UPLC system (Milford, MA) interfaced to an AB Sciex QTrap 5500 (Framingham, MA) mass spectrometer with a Turbo V™ source. Waters Acquity UPLC Console 1.50 software was used to control the UPLC and Analyst 1.6 software by AB Sciex was used to control the mass spectrometer.

LC-MS/MS Conditions

The mass spectrometer conditions were optimized for CPP by directly infusing a 100 ng/mL solution of neat CPP into the mass spectrometer and using the compound optimization setting in the software. Temperature, source gas, curtain gas, ion spray voltages, and collision gas parameters were optimized manually. This process was repeated for the internal standard using a stock solution of cAMP in 20 mM HFBA in 99:1 water:AcN at a concentration of 4.5 µg/mL. The precursor to product ions (Q1 → Q3) selected for the analyte, CPP, were m/z 252.958 → 207.100, 252.958 → 123.000, and 252.958 → 83.000, shown in **Figure 1A**. The precursor to product ions (Q1 → Q3) selected for the internal standard, cAMP, were m/z 334.955 → 136.033, 334.955 → 119.050, and 334.955 → 293.925, shown in **Figure 1B**.

For the LC-MS/MS experiments, the analyte and IS were separated on a Waters Acquity UPLC BEH C18 column (100 × 2.1 mm ID, 1.7 µm) coupled with a Waters Acquity UPLC BEH C18 VanGuard pre-column with (5 × 2.1 mm ID). Mobile phase A was 20 mM HFBA in water and mobile phase B was 20 mM HFBA in acetonitrile. An aliquot of 7 µL of each sample was injected into the column. The following gradient was used to separate the analyte and IS (time/minute, % mobile phase B): (0, 1), (4, 6), (4.1, 95), (5.5, 95), (5.6, 1), (8, 1). The flow rate was set at 0.35 mL/min, the column temperature was 35 °C, and the samples were kept at 10 °C throughout the experiment.

The mass spectrometer was operated in positive ion ESI mode. Multiple reaction monitoring (MRM) mode was utilized to detect the analyte and internal standard. The precursor to product ions used for quantitation for CPP and cAMP were m/z 252.958 → 207.100 and 334.955 → 136.033, respectively. The MRM method contained two periods; CPP was detected in Period 1 (0.0- 2.7 min) and cAMP was detected in Period 2 (2.7-8.0 min). The operational parameters for the mass spectrometer during Period 1 are as follows: curtain gas- 35.0 psi, collision gas- high, ionspray voltage- 4500.0 V, temperature- 625.0 °C, ion source gas 1- 30.0 psi, ion source gas 2- 50.0 psi, declustering potential- 81.0 V, entrance potential- 10.0 V, Q1 resolution- unit, Q3 resolution- low, collision energy- 25 eV, collision cell exit potential- 18 V. The operational parameters for the mass spectrometer during Period 2 are as follows: curtain gas- 55.0 psi, collision gas- high, ionspray voltage- 4500.0 V, temperature- 675.0 °C, ion source gas 1- 30.0 psi, ion source gas 2- 50.0 psi, declustering potential- 86.0 V, entrance potential- 10.0 V, Q1 resolution- unit, Q3 resolution- low, collision energy- 25 eV, collision cell exit potential- 18 V. Peak area ratios of CPP and IS were calculated manually and with AB Sciex MultiQuant 2.1 software by generating calibration curves employing linear or quadratic fits with 1/x weighting. Parameters obtained from these calibration curves were used to back-calculate CPP concentration in mouse plasma.

Immediately following all LC-MS runs with HFBA, the column was flushed with 95% acetonitrile or methanol for at least 10 minutes to remove residual HFBA. The HFBA mobile phase solvents were removed from the LC system and the system was purged and primed with non-HFBA containing solvents to remove residual HFBA.

Method Validation

Linearity, Accuracy, Precision, Sensitivity—Linearity, precision, and accuracy were evaluated using the calibration curve. Linearity was evaluated by the R^2 value on the calibration curve. Accuracy is defined as the closeness between measured and true values and was assessed by the percent relative error (RE), which is calculated as [(actual amount-measured amount)/actual amount] x 100. Precision was assessed by the percent coefficient of variance (CV), which is calculated as [standard deviation of measurements / mean] x 100. Intra-day precision and accuracy were assessed by QC samples equivalent to each point on the calibration curve. The limit of detection (LOD) was tested by injecting samples of neat CPP in water and determining the concentration at which the analyte signal is five times larger than the blank sample. For the LOD study, serial dilutions of neat CPP stock solution in pure water were used. The lower limit of quantitation (LLOQ) was determined from peak areas of the analyte and IS in plasma and defined as the lowest concentration in which the peak area of the sample is five times greater than the area of the blank.

Specificity and Matrix Effects—For this study, the most prominent transition for the analyte and IS were used for quantification; however, three daughter ions for both the analyte and IS were selected and monitored to ensure specificity of the experiment. Solvent calibrations and plasma calibration samples were prepared for evaluating the matrix effects. The matrix calibration samples were prepared by spiking 25 μ L of plasma with 10 μ L of 2 ng/ μ L CPP solution and 10 μ L of 0.5 ng/ μ L cAMP solution. The comparable solvent calibration sample was prepared by spiking 25 μ L of water with 10 μ L of 2 ng/ μ L CPP solution and 10 μ L of 0.5 ng/ μ L cAMP solution. Plasma and solvent calibration samples were dried down and resuspended in 50 μ L of diluent. The matrix effect was calculated as [(the ratio of the peak area of the analyte in post- extraction matrix/the peak area of diluent spiked with the analyte)- 1] x 100 (n=3).

Recovery—Percent recovery was calculated from peak areas of spiked samples and post-preparation spiked samples (n=6). Samples were prepared according to the described SPE procedure and spiked with 20 ng of analyte and 5 ng of IS pre- or post-preparation, dried down, and resuspended in 50 μ L diluent before analysis. Extraction recovery was calculated as [the ratio of peak area of the analyte extracted from the biological matrix/ the peak area of diluent spiked with the analyte] x 100. The recovery of the IS was assessed with the same method.

Stability—Due to the small sample volumes used in this experiment, the entire sample was consumed during each analysis; therefore, the autosampler stability was the most relevant to the experiment. Autosampler stability of the plasma matrix was tested by running freshly prepared samples of 1.0, 10.0, 20.0, and 30.0 ng CPP and 5.0 ng IS in plasma (n=3) immediately and after 11 hrs.

In Vivo Pharmacokinetic Study

CPP solutions for injection were made from CPP stock solution diluted in saline. Injections were given to mice of the 129/SvJ x C57BL/6J background that were between 4-8 weeks of age. Whole blood and brain tissue were collected from animals after decapitation under deep

general anesthesia with isoflurane. For pharmacokinetic study bolus tail vein intravenous (i.v.) injections and intraperitoneal (i.p.) injections were examined. Injection volume was 5 ml/kg for i.v. and 10 ml/kg for i.p. Dosages of i.v. injections included 0.25, 0.5, 1.0, and 2.0 mg/kg, samples were collected 45 minutes following injection, and there were five replicates for each dose. Individual animals were used for a time point study, animals were sacrificed at 10, 15, 30, 45, 60, and 90 minutes following i.v. injection, and there were three or more replicates for each time point. Dosages of i.p. injections included 3.0 and 9.0 mg/kg, samples were collected 60 minutes following injection, and there were five replicates for each dose. Individual animals were used for a time point study, animals were sacrificed at 45, 60, 75, and 90 minutes following i.p. injection, and there were three or more replicates for each time point.

The pharmacokinetic parameters of CPP including maximum concentration ($C_{\max}$) and time point of maximum concentration ($T_{\max}$) were acquired for both plasma and brain tissue following i.v. and i.p. injections. Elimination half-time ($t_{1/2}$) was determined by fitting the concentration-time profiles of plasma and brain tissue after i.v. injection to a one-phase exponential decay using Prism5 (GraphPad Software, Inc., La Jolla, CA). Pharmacokinetic parameters are reported as mean $\pm$ SEM.

Results and Discussion

Method Validation

Linearity, Accuracy, Precision, Sensitivity—The higher end of the plasma calibration curve range began to saturate the detector on the mass spectrometer; therefore, a quadratic fit was used with an R^2 value of 0.99. Intra-day precision and accuracy was calculated for all points on the calibration curve, shown in **Table 1**. Precision was assessed by the CV and is defined as the closeness of measurements of the same concentration. Accuracy was assessed by the RE between measured and nominal concentrations. CV and RE values were less than 15%. Due to the small sample volumes used in this experiment, the entire sample was consumed during each analysis; therefore, the inter-day precision and accuracy is not analyzed. The LOD, the concentration at which the analyte signal is five times larger than the blank sample was determined to be 50 ag on column (5 μ L injection of 10 ag/mL CPP in water). The LLOQ is 0.3 ng for plasma samples.

Specificity and Matrix Effects—Representative chromatograms obtained from blank diluent, neat CPP (20 ng), blank plasma, and plasma spiked 20 ng CPP are shown in **Figure 2**. A peak is occasionally observed in the blank plasma solutions at a retention time overlapping with that of CPP. This peak is believed to be potassiated HFBA. Protonated CPP has an exact mass of 253.094785 while potassiated HFBA has a mass of 252.949635 ($m = 0.145$ Da) and the two compounds have similar transition masses. The inherent nature of the Q- trap as a low resolution mass spectrometer does not allow for differentiation of masses that are this close together, therefore the potassiated HFBA generates a peak in the blanks samples. This effect has been accounted for in the calibration curves as the intercepts do not go through zero. The effects of the plasma matrix on ion suppression were evaluated at relevant concentration levels (20 ng CPP). A suppression of $-12.27 \pm 9.45\%$ was

observed for CPP in plasma and a slight enhancement of $+4.60 \pm 11.70\%$ was observed for IS.

Recovery—Recovery of CPP was determined by dividing the peak area of the sample with CPP spiked in pre-preparation by the peak area of the sample with CPP spiked in post-preparation in 3 replicates each in the plasma matrix. This method was also used to analyze the recovery of the IS. Average recovery for CPP in plasma was $58.21 \pm 13.40\%$ and average recovery for IS in plasma was $88.04 \pm 12.59\%$.

Stability—Autosampler stability (10°C) was determined for the analyte and IS in plasma at relevant concentrations. The stability is shown in the form of percentage of relative concentration of samples run after 11 hours to samples run initially (mean $\pm$ SD). The results are summarized in **Table 2**.

Pharmacokinetics

The described method was used to assay CPP concentrations in mice following systemic administration. I.V. administration of CPP at a series of doses indicated a linear relationship between the dose and concentration for both plasma and brain tissue over the concentration range examined (**Figure 3A**). I.P. administration at two doses revealed a similar relationship (**Figure 3B**). We calculated the brain to plasma concentration ratio using the 1 mg/kg dose for i.v. administration and 3 mg/kg dose for i.p. administration, yielding 0.07 ± 0.01 and 0.06 ± 0.01 , respectively. A detailed concentration-time profile was performed using i.v. administration of 1 mg/kg CPP (**Figure 4**). $C_{\max}$ for plasma was 2113 ± 202 ng/ml ($n=3$) at $T_{\max}$ (10 minutes), which was the first time point assayed. $C_{\max}$ for brain tissue was 23.9 ± 6.0 ng/g ($n=4$) at $T_{\max}$ (15 minutes), also the first time point assayed. The elimination of CPP was modeled as one-phase exponential decay. The $t_{1/2}$ of CPP in plasma was 8.8 minutes and the $t_{1/2}$ of CPP in tissue was 14.3 minutes. The concentration-time profile of CPP following i.p. injection of 9 mg/kg CPP was assessed over a relevant timeframe (**Figure 5**). Following i.p. administration $C_{\max}$ for CPP in plasma was 1259 ± 177 ng/ml ($n=8$) at $T_{\max}$ (60 minutes), while $C_{\max}$ for CPP in brain tissue was 87 ± 32 ng/g ($n=5$) at $T_{\max}$ (45 minutes).

Conclusions

A reliable and sensitive ion-pair LC-MS/MS assay for the determination of CPP in mouse plasma and tissue was developed. Using this method we report a pharmacokinetic analysis of CPP, a competitive antagonist of the NMDA receptor that is routinely used to investigate the role of NMDA receptors in brain function. Importantly, this assay does not require a tritiated compound, which is not readily available, it is 100 times more sensitive than the derivatization method presented by Patel et al. [26], and it is suitable for extensive pharmacokinetic studies. The pharmacokinetics of CPP presented here will inform research into the myriad of NMDA-dependent processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MS/MS spectra showing A) CPP fragment ions 207.100, 123.000, and 83.000 and B) fragment ions 136.033, 119.050, and 293.925 monitored in the MRM experiments.

Figure 2. Representative chromatograms obtained from A) blank diluent, B) 20 ng neat CPP, C) blank plasma with inlay of zoomed in chromatogram, and D) plasma spiked with 20 ng CPP. The green line in each chromatogram separates the two periods of the MS method. Period 1 measures CPP while period 2 measures IS.

Figure 3. CPP dose-concentration relationships. A) Animals were administered CPP by i.v. injection, and were sacrificed 45 minutes later in order to sample plasma and brain tissue. B) Animals were administered CPP by i.p. injection, and were sacrificed 60 minutes later in order to sample plasma and brain tissue. The left axis presents plasma concentration and the right axis presents tissue concentration. Symbols and error bars are mean $\pm$ SEM

Figure 4. Concentration-time plot for plasma and brain tissue following i.v. injection of 1 mg/kg CPP. Non-linear regression was performed with an on-phase exponential decay, for plasma $r^2=0.964$, for brain tissue $r^2=0.774$. Symbols and error bars are mean $\pm$ SEM.

Figure 5.
Concentration-time plot for plasma and brain tissue following i.p. injection of 9 mg/kg CPP.
Symbols and error bars are mean $\pm$ SEM.

Table 1

The intra-day precision (CV) and accuracy (RE) of the LC-MS/MS method used to quantitate CPP in mouse plasma

	N	Nominal Amount (ng)	Measured Amount (mean±SD, ng)	CV (%)	RE (%)
Plasma	7	0.5	0.6±0.0	3.32	8.38
	9	2.5	2.4±0.2	8.70	2.65
	7	10.0	9.9±0.6	6.41	0.70
	9	50.0	49.5±4.9	9.82	0.97
	7	100.0	100.9±8.1	8.07	0.91
	8	150.0	149.6±13.8	9.25	0.28

Table 2

Autosampler stability of CPP and IS at all concentrations used to generate the calibration curves in plasma. The stability is shown in the form of percentage of relative concentration of samples run at several time points

Matrix	Compound	Amount (ng)	Stability (%)
Plasma (11 hr)	CPP	1.0	97.33 $\pm$ 2.04
		10.0	98.65 $\pm$ 9.01
		20.0	93.43 $\pm$ 2.79
		30.0	84.98 $\pm$ 9.68
	IS	5.0	88.50 $\pm$ 2.44