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Validation and application of a liquid chromatography–tandem mass spectrometric method for the determination of SCH 211803 in rat and monkey plasma using automated 96-well protein precipitation

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Abstract

A rapid, sensitive, specific, accurate, and reproducible automated liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the quantitative determination of 1′-(2-amino-3-methylbenzoyl)-4-[[[(3-chlorophenyl)sulfonyl]phenyl]methyl]-1,4′-bipiperidine hydrochloride (SCH 211803) in plasma has been developed. The method was validated in rat and monkey plasma over the concentration range of 0.5–250 ng/ml using $^{2}H_4$-SCH 211803 as the internal standard (IS). Automated 96-well plate protein precipitation (PP) with acetonitrile (ACN) was used for sample processing. The method employed a Betasil C18 column with a fast gradient for the separation of analyte and internal standard from the plasma matrix and a triple quadrupole mass spectrometer operated in positive ion multiple reaction monitoring (MRM) mode for detection. The method was used for the determination of SCH 211803 plasma concentrations to support pre-clinical studies.

Keywords: Protein precipitation; SCH 211803

1. Introduction

1′-(2-amino-3-methylbenzoyl)-4-[[[(3-chlorophenyl)sulfonyl]phenyl]methyl]-1,4′-bipiperidine hydrochloride (SCH 211803) is a selective antagonist of cholinergic muscarinic M_2 receptors and is a candidate for the treatment of Alzheimer's disease (AD). The current treatment of AD utilizes acetylcholinesterase inhibition to increase acetylcholine (ACh) concentrations in the synaptic cleft [1,2]. The use of SCH 211803 is an alternative strategy, which increases intrasynaptic acetylcholine levels by inhibiting presynaptic M_2 autoreceptors [3–5]. Animal studies have shown that SCH 211803 improved both learning and consolidation memory in rats. SCH 211803 also enhanced spatial memory and short-term memory in monkeys.

To support pre-clinical toxicokinetic and pharmacokinetic studies, an automated liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the determination of SCH 211803 was developed and validated in rat and monkey plasma. The effect of different sample processing procedures on the determined concentrations of SCH 211803 in plasma samples from a drug discovery study was also investigated. Using the 96-well format, one analyst extracted up to 96 samples (one block) in approximately 20 min or 254 samples (three blocks) in 1 h. These results represented an increase in throughput when compared with a conventional liquid–liquid (LL) extraction method previously used for pre-clinical sample analysis in which one analyst extracted 20 samples in 20 min.

2. Experimental

2.1. Materials and reagents

The analyte, SCH 211803 and its isotopically labeled internal standard (IS), $^{2}H_4$-SCH 211803 (see Fig. 1), were

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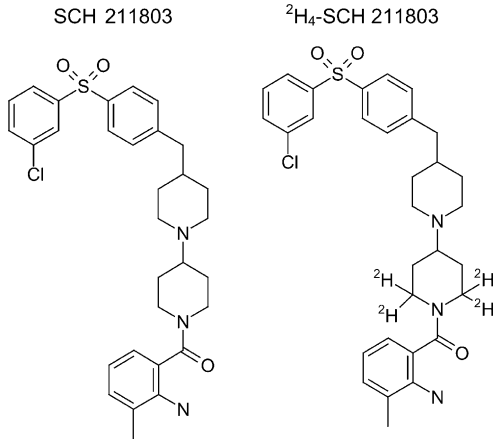

Fig. 1. Chemical structures of SCH 211803 and the IS, $^2H_4$-SCH 211803.

synthesized by Schering–Plough Research Institute (Kenilworth, NJ, USA). Rat and monkey plasma, with EDTA as the anti-coagulant, were purchased from Bioreclamation Inc. (Hicksville, NY, USA). OPTIMA Grade acetonitrile (ACN) and OPTIMA Grade methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (minimum of 95%) was obtained from Sigma (St. Louis, MO, USA). Sequanal grade trifluoroacetic acid was purchased from Pierce Chemical Company (Rockford, IL, USA). The Milli-Q water used in this study was purified in-house using an A10 Millipore water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Preparation of standards and quality control samples

Two separate weighings of SCH 211803 were made to prepare stock solutions at 0.5 mg/ml in methanol. One stock solution was used to prepare working solutions for the calibration curve standards (STD) and was called the STD stock solution. The other stock solution was used to prepare working solutions for quality control (QC) samples and was called QC stock solution. Standard spiking solutions at 2.00, 4.00, 10.0, 40.0, 100, 400, and 1000 ng/ml were prepared from serial dilutions of the STD stock solution in methanol:water (2:1). QC spiking solutions at 0.100, 0.300, 25.0, and 40.0 g/ml were prepared from serial dilutions of the QC stock solution in methanol:water (2:1). All of the solutions were stored in 20 ml vials at 4 °C. Calibration standards were prepared by spiking 100 μl of blank rat plasma with 25 μl of the standard working solutions of SCH 211803 to give nominal concentrations of 0.500, 1.00, 2.50, 10.0, 25.0, 100, and 250 ng/ml. Therefore, the lower limit of quantitation (LLOQ) for this assay is 0.5 ng/ml and the upper limit of quantitation (ULOQ) is 250 ng/ml. For each validation run, the calibration curve standards were prepared fresh and in duplicate from standard spiking solutions. QC samples were prepared in 10 ml pools by spiking blank plasma with 50 μl of the appropriate QC working solution to give nominal concentrations of 0.500 (QC LLOQ), 1.50 (QC low), 125 (QC medium), and 200 ng/ml (QC high). An over-range QC pool was also prepared at 400 ng/ml. The QC samples were stored at −80 °C until analyzed.

One weighing was made to prepare the internal standard stock solution at 0.5 mg/ml in methanol. Then, an intermediate IS stock solution at 5 μg/ml was prepared by dilution of 100 μl of IS stock solution into 10.0 ml methanol:water (2:1). IS working solution at 5.00 ng/ml was prepared by dilution of 250 μl of IS intermediate stock solution into a 250 ml acetonitrile.

2.3. Sample preparation

Samples were prepared using an automated protein precipitation (PP) method with a TOMTEC™ Quadra 96 Model 320 liquid handling system (TomTec, Hamden, CT). Frozen control plasma and QC pools were thawed at room temperature prior to use. Working stock solutions were also removed from the refrigerator and warmed to room temperature before use. One hundred microlitres of blank plasma or QC pools were transferred to cluster tubes (Costar Corporation, Cambridge, MA) and arranged into a 96-well format. Twenty-five microlitres of standard working solutions were added into 100 μl of blank plasma to make the fresh calibration curve standards. Twenty-five microlitres of methanol:water (2:1) were added into 100 μl of blank plasma or QC pools to prepare the blank and QC samples. Then, 250 μl of IS working solution were added to the samples except for the double blanks in which 250 μl ACN were added. The samples were then covered and vortex-mixed for 5 min at medium speed. After centrifugation at 1845 × *g* for 10 min, 250 μl of the supernatant was transferred into 1 ml 96-well plates and 250 μl of mobile phase A were added to each well. After mixing, the 96-well plate was covered and transferred to the autosampler. Fifty microlitres of the processed sample were injected onto the LC–MS/MS system.

2.4. Liquid chromatography–mass spectrometry

The liquid chromatographic system consisted of a Perkin-Elmer 200 Series autosampler (The Perkin-Elmer Corporation, Norwalk, CT, USA), two Shimadzu LC-10AD VP liquid chromatographic pumps, a Shimadzu SCL-10A VP system controller, a Shimadzu CTO-10A VP column oven, and a Shimadzu DGU-14A degasser (Shimadzu Corporation, Columbia, MD, USA).

Separation of the analyte and IS was achieved using a 2 mm × 100 mm, 5 μm Keystone Betasil C18 column and a 2 mm × 20 mm, 5 μm Keystone Betasil C 18 guard column (Keystone Scientific Inc., Bellefonte, PA, USA) with a flow rate of 0.3 ml/min. The column oven temperature was set at 60 °C. The following linear gradient elution was used: start with 55% B, ramp to 75% B in 3 min, jump to 100% B at 3.01 min and hold at 100% B until 5.50 min, back to 55% B at 5.55 min, and stop at 6 min. Mobile phase A was an aqueous solution with 0.2% formic acid and mobile phase B

was acetonitrile/methanol (1:1) with 0.2% formic acid. The autosampler wash solution was methanol with 0.2% TFA. A 50 μl aliquot was injected from 500 μl total processed sample volume.

The analyte and IS were detected using a PE Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ionspray interface (AB/MDS/Sciex, Concord, Ontario, Canada). The mass spectrometer was operated in the positive ion multiple reaction monitoring (MRM) mode. The following MRM transitions were monitored for the analyte and IS:SCH 211803, *m/z* 566.2 to *m/z* 134.1; $^{2}H_{4}$-SCH 211803, *m/z* 572.2 to *m/z* 134.1. The dwell time for each transition was 200 ms with a 2 ms pause between scans. The total cycle time was 404 ms. The turbo ionspray probe temperature was set at 500 °C with an auxiliary nitrogen gas flow of 8.0 l/min. Curtain, nebulizer, and collision gases were set at 8, 10, and 6, respectively. UHP nitrogen was used except for the nebulizer gas, for which zero grade air was used. The ionspray voltage was set at 4200 V. The collision energy was set at 49 V.

Calibration of the instrument was performed by infusing a 10-fold dilution of a polypropylene glycol (PPG) standard tuning solution (AB/MDS/Sciex, Concord, Ontario, Canada) at a flow rate of 10 μl/min into the mass spectrometer. The analyte and IS signals were optimized while infusing a solution containing 5 μg/ml of the analyte and IS at 10 μl/min into a flow of 190 μl/min of 80% mobile phase B. Peak widths were approximately 0.7 amu at half height for both quadrupole one and quadrupole three.

Sample Control Version 1.4 software (AB/MDS/Sciex, Concord, Ontario, Canada) was used for instrument control and data acquisition. TurboQuan™ Version 1.0 software (AB/MDS/Sciex, Concord, Ontario, Canada) was used for peak integration and quantitative analysis.

2.5. Validation and sample analysis procedures

Validation was carried out according to the US Food and Drug Administration (FDA) and pharmaceutical industry guidelines [6]. A complete method validation was performed for each toxicology species. The within- and between-run accuracy and the precision of the method were assessed with three main validation runs. Each main validation run contained duplicate calibration curve standards at 10 concentrations, quality control samples at 4 concentrations ($n = 6$ at each concentration, including LLOQ). Each run also contained a minimum of two blank plasma samples with IS and two blank plasma samples without IS (not used in the regression). Six different lots of blank plasma were screened for endogenous interference. Additional validation runs and sample analysis runs contained duplicate calibration curve standards at ten concentrations, QC samples at three concentrations ($n = 3$ at each concentration, not including LLOQ), and a minimum of four blanks (two with IS and two without IS).

The stability of SCH 211803 was assessed under various storage conditions using QC samples ($n = 6$) prepared at the low and the high concentrations of 1.5 and 200 ng/ml.

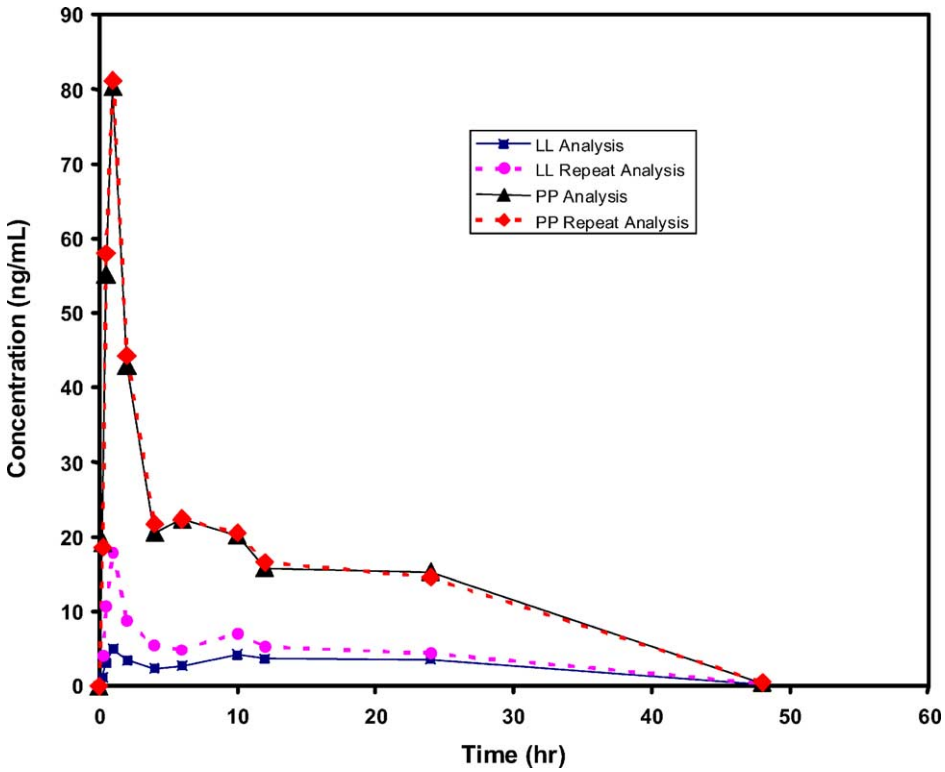

Fig. 2. Time–concentration profiles of one subject in a non-GLP drug discovery study using liquid–liquid (LL) extraction and protein precipitation (PP).

3. Results and discussion

3.1. Sample preparation method

Initially, a liquid–liquid extraction method with hexane as the extraction solvent was used for the quantitation of SCH 211803 in monkey and rat plasma. The method passed full validation with very good precision and accuracy. Stability experiments also passed acceptance criteria with the spiked QC samples in both rat and monkey plasma. Nevertheless, problems were encountered when analyzing samples from a non-GLP drug discovery study. Upon sample reprocessing and repeat analysis, the concentrations were found to be up to five times higher than the original values. Following further investigation, it was found that the sample concentrations increased with each successive freeze–thaw cycle, until reaching a plateau value. Furthermore, this phenomenon was only observed for study samples from animals and not for QC samples fortified with SCH 211803. As an explanation for these observations, we postulated that since SCH 211803 is very highly bound to plasma proteins (>99%), perhaps hexane was not disrupting all of the protein binding. The temperature change in each freeze–thaw cycle, however, gradually denatured proteins and released analyte from protein interactions. Based upon this hypothesis, a protein precipitation method using ACN as the precipitation reagent was used for sample preparation to free the analyte from tight protein binding. Fig. 2 shows the time–concentration

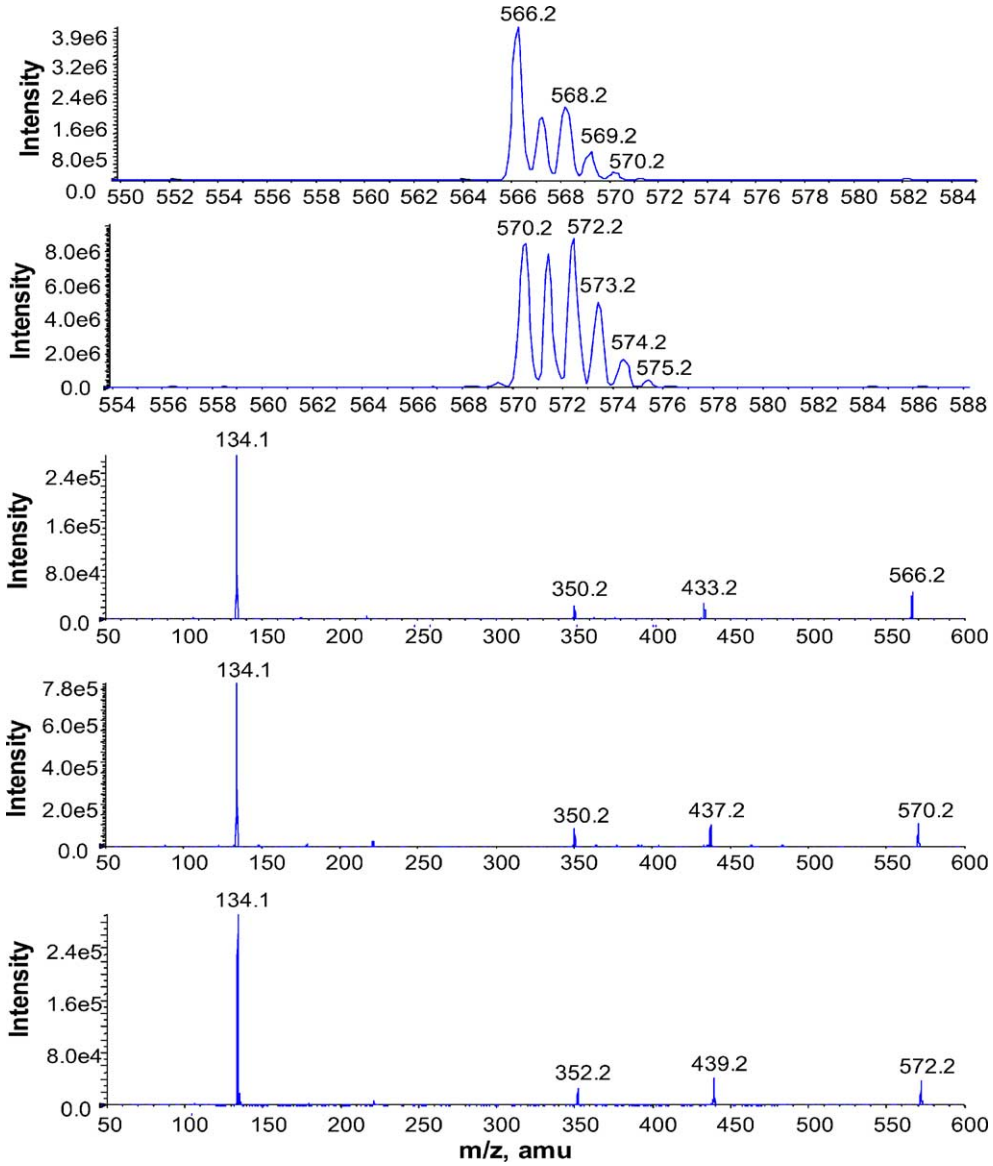

Fig. 3. Mass spectra of SCH 211803 and its IS. From top to bottom: Q1 scan mass spectrum of SCH 211803, Q1 scan mass spectrum of IS, product ion scan spectrum of SCH 211808 (parent ion at *m*/*z* 566.3), product ion spectrum of IS (parent ion at *m*/*z* 570.3), and product ion spectrum IS (parent ion at *m*/*z* 572.3).

profiles of one monkey subject in a drug discovery study using the liquid–liquid extraction method and also the protein precipitation method. For liquid–liquid extraction, reanalysis values were higher than the original values. For the protein precipitation method, reanalysis values were consistent with the original values. Furthermore, concentrations obtained from the protein precipitation method were higher than those obtained from the liquid–liquid extraction method.

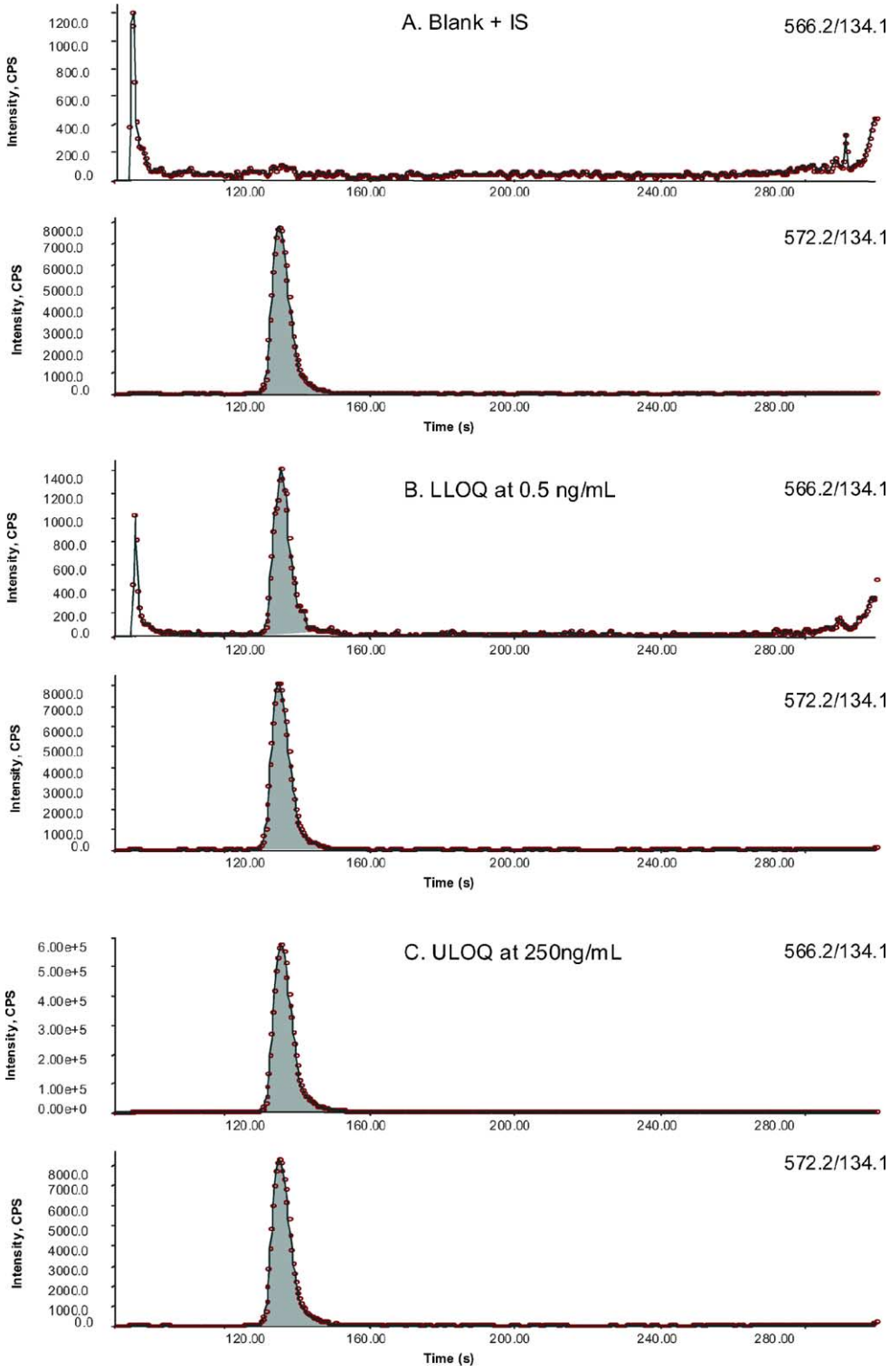

Fig. 4. Representative chromatograms from the method validation in monkey plasma: (A) a blank plasma sample with IS, injected immediately after QC high at 200 ng/ml, (B) an LLOQ at 0.500 ng/ml, and (C) a ULOQ at 250 ng/ml. The following MRM transitions were monitored for the analyte and IS:SCH 211803, *m/z* 566.2 to *m/z* 134.1; $^{2}H_{4}$-SCH 211803, *m/z* 572.2 to *m/z* 134.1.

3.2. Liquid chromatography and mass spectrometry

The full scan and product ion mass spectra of SCH 211803 and $^{2}H_4$-SCH 211803 are shown in Fig. 3. In the full scan spectra, the isotopic distributions of the protonated molecular ion clusters for both the analyte and the IS are shown. The isotopic distribution pattern of the IS is not as expected, most likely indicating that the IS is not pure $^{2}H_4$-SCH 211803 but rather a mixture of $^{2}H_4$-, $^{2}H_5$-, $^{2}H_6$-, and $^{2}H_7$-SCH 211803.

In the product ion spectra, the dominant product ion for both SCH 211803 and $^{2}H_4$-SCH 211803 is *m*/*z* 134.1. The MRM transition that was used to monitor the analyte was *m*/*z* 566.2 to *m*/*z* 134.1. Since the isotopic distribution pattern of SCH 211803 shows approximately 4% contribution at *m*/*z* 570.2, the transition *m*/*z* 572.2 to *m*/*z* 134.1 was selected for the IS to avoid contribution from the analyte to the IS response.

Fig. 4 shows three representative chromatograms from monkey plasma: (A) a chromatogram of a blank plasma sample with IS, injected immediately after QC high at 200 ng/ml, (B) a chromatogram of calibration standard at 0.500 ng/ml (LLOQ), and (C) a chromatogram of calibration standard at 250 ng/ml (ULOQ). The retention times of both SCH 211803 and the IS were approximately 2.3 min. The chromatogram at the LLOQ shows good signal-to-noise ratio. Six blank plasma lots were screened to evaluate the specificity of the method. There were no endogenous components in the blank plasma that interfered with the analyte as demonstrated by the representative blank chromatogram shown in Fig. 4. This blank was injected after a QC high sample demonstrating very little carryover of analyte from previous injections.

Table 1
Calibration curve parameters for SCH 211803 in rat and monkey plasma

Matrix	Run number	Correlation coefficient (*r*)	*a*[a]	*b*[a]	*c*[a]
Rat plasma	Run 1	0.999	−1.40E-04	0.320	2.26E-03
	Run 2	1.00	−2.00E-04	0.342	7.64E-03
	Run 3	1.00	−8.80E-05	0.314	6.98E-03
Monkey plasma	Run 1	1.00	−1.40E-04	0.382	0.0206
	Run 2	1.00	−2.10E-04	0.393	0.0143
	Run 3	1.00	−1.50E-04	0.346	0.0233

[a] $y = ax^2 + bx + c$, where y is the peak area ratio of SCH 211803 to IS; x is the concentration of SCH 211803; and a, b and c are curve parameters of the calibration curve.

3.3. Regression, accuracy, and precision

A 1/*y* weighted quadratic regression gave an excellent fit for the concentration/detector response relationship for SCH 211803 in both rat and monkey plasma. A representative calibration curve from a validation run in monkey plasma is shown in Fig. 5. For each validation run, the correlation coefficient (*r*) of the calibration curve was ≥0.999 in both rat and monkey plasma. Summaries of the calibration curve parameters are shown in Table 1.

The performance of the assay was determined by assessing the precision (%CV) and accuracy (% difference from nominal) for QC samples in replicates of six at four concentration levels (Tables 2 and 3). Current FDA-recommended acceptance criteria for precision and accuracy are ±15% at the low, medium, and high QC levels and ±20% at the LLOQ QC level. For monkey plasma, the precision ranged

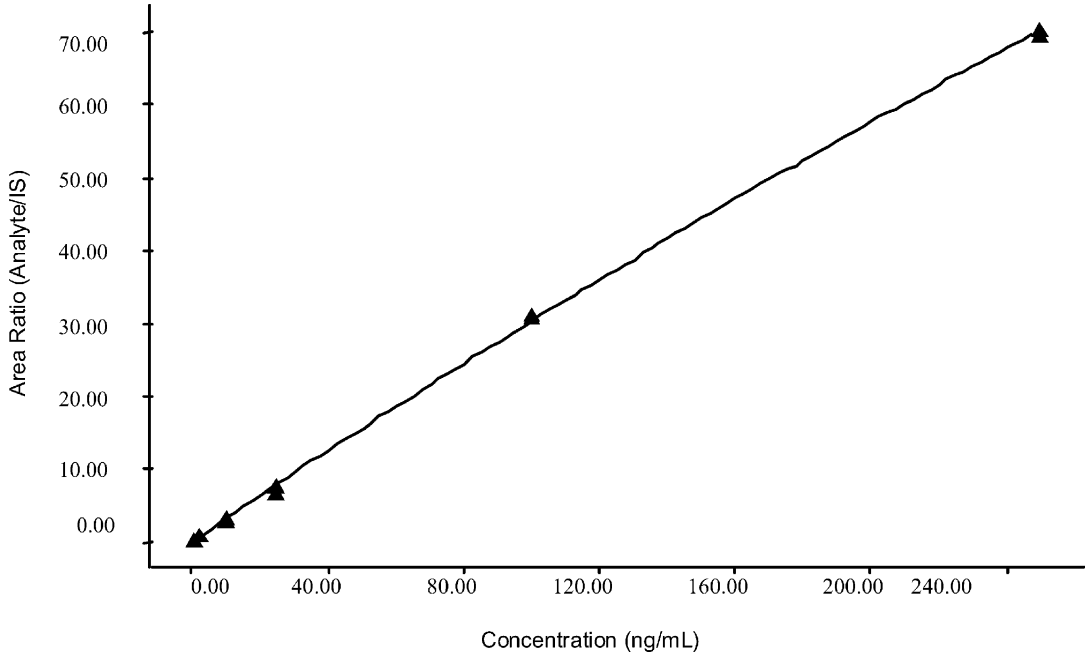

Fig. 5. A representative calibration curve from the method validation in monkey plasma.

Table 2
Quality control sample concentrations of SCH 211803 in monkey plasma (within- and between-run precision and accuracy)

Run number	Statistics	QC LOQ 0.500 (ng/ml)	QC low 1.50 (ng/ml)	QC medium 125 (ng/ml)	QC high 200 (ng/ml)
Run 1	Mean	0.464	1.50	117	190
	%CV	6.12	3.10	2.39	1.92
	n	6	6	6	6
	Mean % difference	−7.20	0	−6.40	−5.00
Run 2	Mean	0.495	1.44	113	191
	%CV	10.6	4.46	2.02	0.840
	n	6	6	6	6
	Mean % difference	−1.00	−4.00	−9.60	−4.50
Run 3	Mean	0.437	1.44	115	185
	%CV	7.50	2.27	1.96	1.43
	n	06	6	6	6
	Mean % difference	−12.6	−4.00	−8.00	−7.50
Between-run	Between-run mean	0.465	1.46	115	189
	Between-run %CV	9.54	3.72	2.57	2.01
	n	18	18	18	18
	Between-run mean % difference	−7.00	−2.67	−8.00	−5.50

from 0.840 to 10.6% and accuracy ranged from −12.6 to 0%. For rat plasma, the precision ranged from 2.15 to 12.7% and accuracy ranged from −12.4 to 3.33%. The results indicate that the method was accurate and precise.

3.4. Stability results

The stability of SCH 211803 under various storage conditions was evaluated by comparing the concentrations of low and high stability QC samples to their respective nominal concentrations. For instance, to evaluate the stability of SCH 211803 stored at room temperature for 24 h, the low and high QC samples were left in at room temperature for 24 h and then processed and analyzed. SCH 211803 was deemed to be stable if the precision and accuracy of those stability QC samples were no greater than 15%.

SCH 211803 was stable in monkey plasma for at least 24 h at room temperature, 349 days at −80 °C, and after 10 freeze–thaw cycles. SCH 211803 was also stable after processing at room temperature and 4 °C for at least 24 h (Table 4).

SCH 211803 was stable in rat plasma for at least 24 h at room temperature, 384 days at −80 °C, and after eight freeze–thaw cycles. SCH 211803 was also stable after processing at room temperature and 4 °C for at least 24 h (Table 5).

Table 3
Quality control sample concentrations of SCH 211803 in rat plasma (within- and between-run precision and accuracy)

Run number	Statistics	QC LOQ 0.500 (ng/ml)	QC low 1.50 (ng/ml)	QC medium 125 (ng/ml)	QC high 200 (ng/ml)
Run 1	Mean	0.507	1.55	126	199
	%CV	12.7	7.56	3.66	9.35
	n	6	6	6	6
	Mean % difference	1.40	3.33	0.800	−0.500
Run 2	Mean	0.438	1.40	120	201
	%CV	5.84	4.89	3.48	2.15
	n	6	6	6	6
	Mean % difference	−12.4	−6.67	−4.00	0.500
Run 3	Mean	0.464	1.51	121	200
	%CV	7.11	3.43	2.63	3.38
	n	6	6	6	6
	Mean % difference	−7.20	0.670	−3.20	0
Between-run	Between-run mean	0.470	1.49	122	200
	Between-run %CV	10.8	6.84	3.85	5.51
	n	18	18	18	18
	Between-run mean % difference	−6.00	−0.670	−2.40	0

Table 4
Stability of SCH 211803 in monkey plasma

Stability	Statistics	QC low 1.50 (ng/ml)	QC high 200 (ng/ml)
Stability at room temperature (24 h)	Mean	1.44	186
	%CV	5.10	1.69
	n	6	6
	Mean % difference	−4.00	−7.00
Stability at −80 °C (349 days)	Mean	1.58	184
	%CV	2.50	1.00
	n	5	6
	Mean % difference	5.33	−8.00
Freeze–thaw stability (10 cycles)	Mean	1.58	196
	%CV	4.07	2.51
	n	6	6
	Mean % difference	5.33	−2.00
Stability of processed samples at room temperature (24 h)	Mean	1.56	196
	%CV	1.54	1.15
	n	6	6
	Mean % difference	4.00	−2.00
Stability of processed samples at 4 °C (24 h)	Mean	1.59	198
	%CV	2.38	1.73
	n	6	5
	Mean % difference	6.00	−1.00

3.5. Recovery

Recovery experiments were performed in both rat and monkey plasma using $^{2}H_4$-SCH 211803. The signal obtained using QC samples spiked with IS at 5.00 ng/ml before processing was compared to the signal obtained for a neat solution of IS at 5.00 ng/ml. The calculated recoveries in monkey and rat plasma were 94.2 and 97.9%, respectively.

3.6. Application of the methods

The validated LC–MS/MS methods were successfully applied to preclinical studies in rats and monkeys. A chromatogram from a Day 0 study sample taken at 2 h after oral dosing at 3 mg/kg in a 1 month oral (gavage) toxicity and toxicokinetic study in monkeys is shown in Fig. 6. A typical time–concentration profile of SCH 211803 following a

Table 5
Stability of SCH 211803 in rat plasma

Stability	Statistics	QC low 1.50 (ng/ml)	QC high 200 (ng/ml)
Stability at room temperature (24 h)	Mean	1.48	198
	%CV	5.83	3.74
	n	6	6
	Mean % difference	−1.33	−1.00
Stability at –80 °C (384 days)	Mean	1.45	194
	%CV	3.80	1.50
	n	6	6
	Mean % difference	−3.33	−3.00
Freeze–thaw stability (eight cycles)	Mean	1.48	197
	%CV	5.54	3.32
	n	6	6
	Mean % difference	−1.33	−1.50
Stability of processed samples at room temperature (24 h)	Mean	1.52	205
	%CV	3.59	2.88
	n	6	6
	Mean % difference	1.33	2.50
Stability of processed samples at 4°C (24 h)	Mean	1.60	213
	%CV	5.63	2.17
	n	6	6
	Mean % difference	6.67	6.50

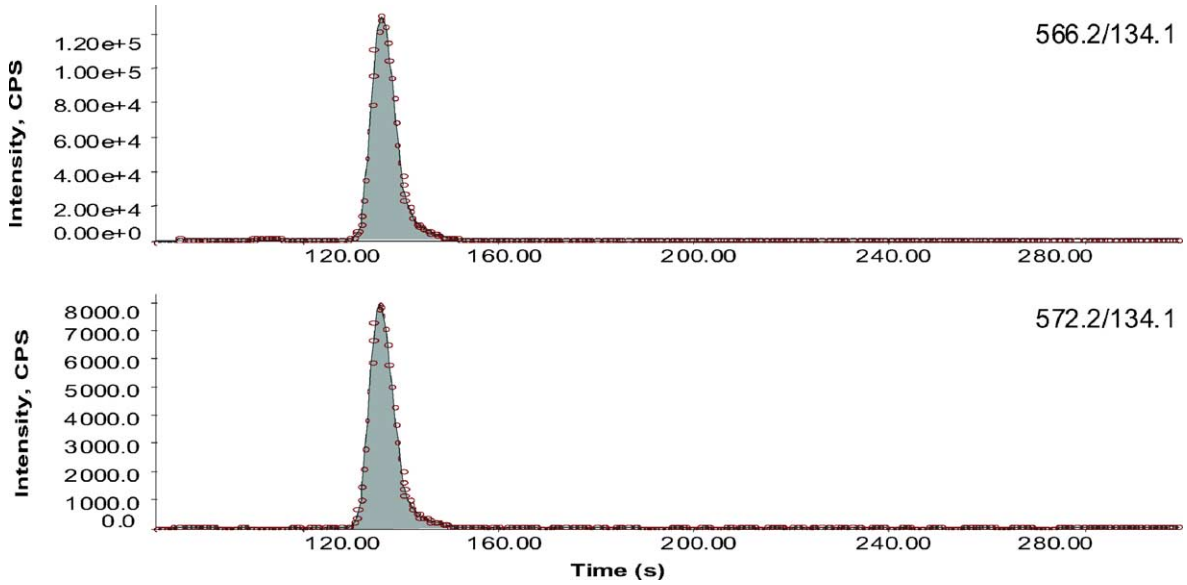

Fig. 6. A chromatogram from a Day 0 study sample taken at 2 h after oral dosing at 3 mg/kg in a 1 month oral (gavage) toxicity and toxicokinetic study. SCH 211803, *m*/*z* 566.2 to *m*/*z* 134.1 (top panel); $^{2}H_{4}$-SCH 211803, *m*/*z* 572.2 to *m*/*z* 134.1 (bottom panel).

3 mg/kg oral dose to a male monkey in that study is presented in Fig. 7. The ULOQ of the assay was high enough to limit the number of repeat analyses due to concentrations above the ULOQ. In addition, the LLOQ was sufficiently low enough so that the terminal phase of the concentration time profiles could be characterized at the lowest doses used in the toxicokinetic studies. A typical time–concentration profile of SCH 211803 following a 2.5 mg/kg oral dose to female rats in a 1 month oral (gavage) toxicity and toxicokinetic study is shown in Fig. 8.

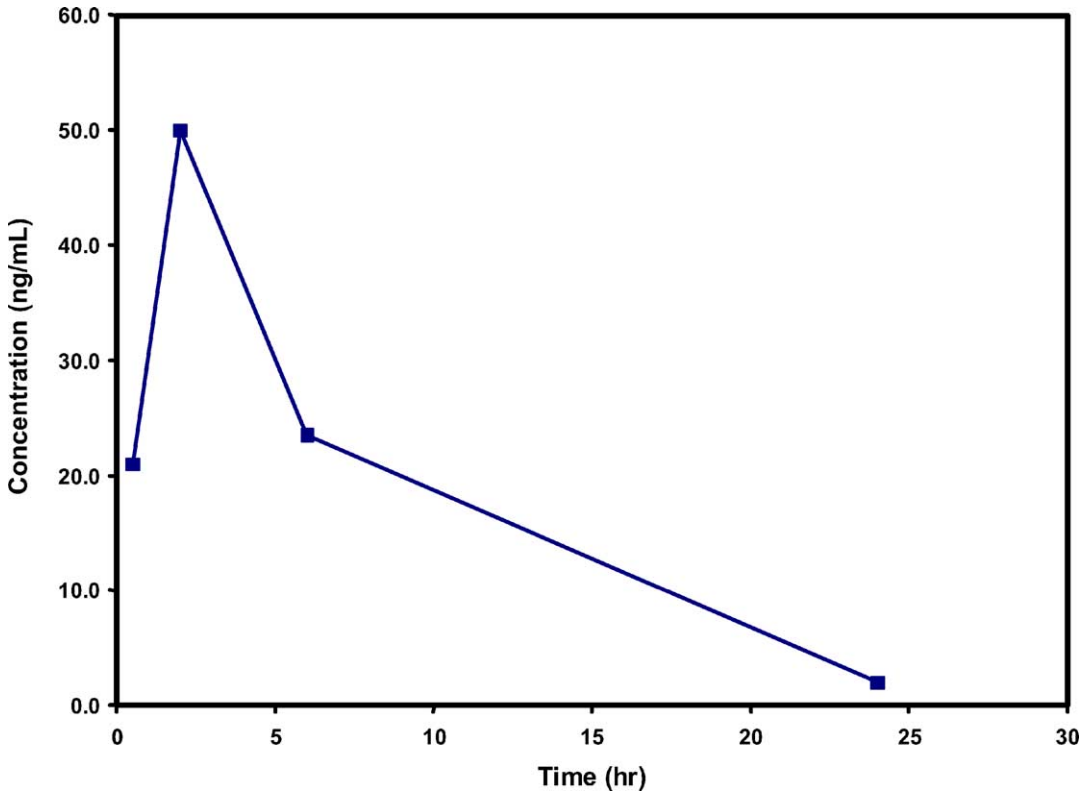

Fig. 7. A typical time–concentration profile of SCH 211803 following a 3 mg/kg oral dose to a male monkey in a 1 month oral (gavage) toxicity and toxicokinetic study.

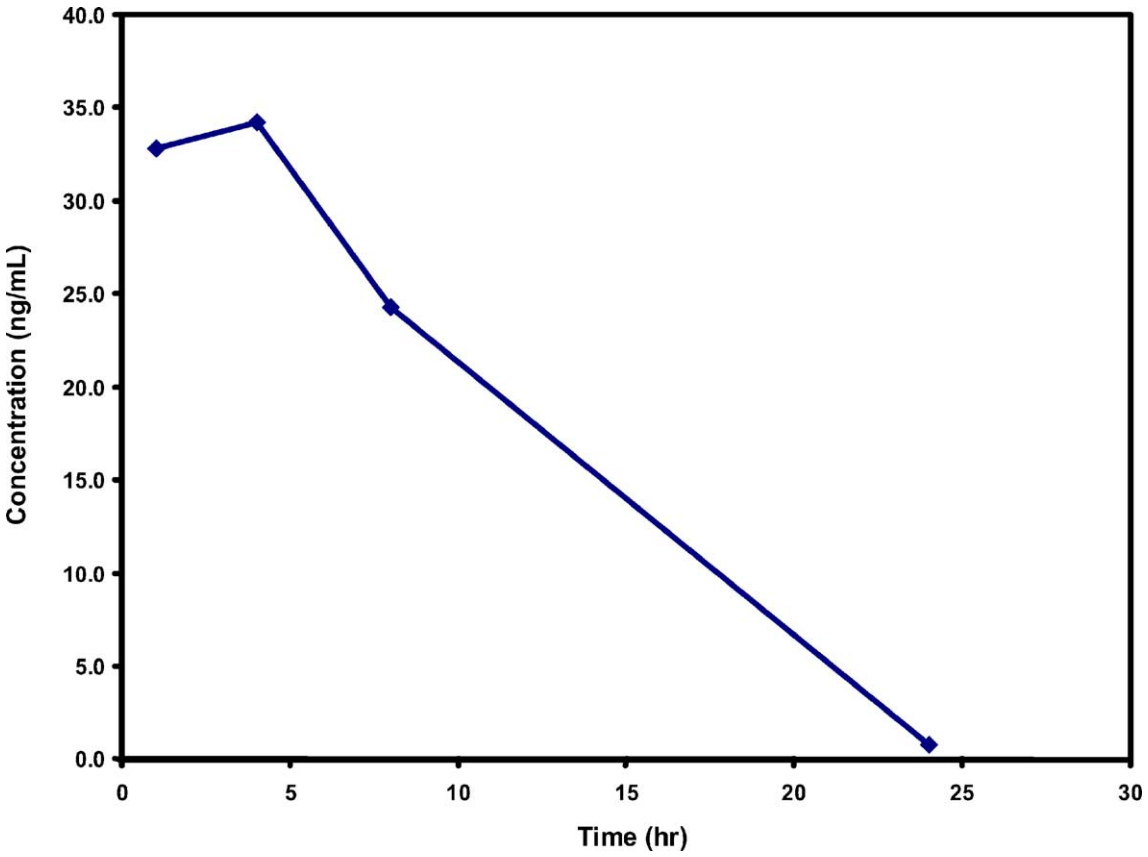

Fig. 8. A typical time–concentration profile of SCH 211803 following a 2.5 mg/kg oral dose to female rats in a 1 month oral (gavage) toxicity and toxicokinetic study.

4. Conclusions

A sensitive LC–MS/MS method for the quantitative determination of SCH 211803 using automated 96-well protein precipitation was validated in both rat and monkey plasma. The calibration curves showed goodness of fit over the concentration range of 0.5–250 ng/ml using a quadratic regression with $1/y$ weighting. Within- and between-run precision and accuracy for calibration standards and QCs met FDA acceptance criteria for bioanalytical method validations. SCH 211803 was stable in rat and monkey plasma during typical sample storage conditions. The validated LC–MS/MS methods were used to support preclinical studies in rats and monkeys.

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