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Validation and application of an automated 96-well solid-phase extraction liquid chromatography–tandem mass spectrometry method for the quantitative determination of SCH 201781 in human plasma

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Abstract

SCH 201781 is a direct thrombin inhibitor recently under study in clinical trials to determine its safety and efficacy for the treatment of venous and arterial thrombosis. In aqueous solution, SCH 201781 exists as three forms, a ring-opened hydrated form and two ring-closed diastereomers. An automated solid-phase extraction LC-MS/MS method that chromatographically separates and measures each form was developed and validated from 1 to 1000 ng/mL in human plasma. For calibration curve standards, within- and between-run precision (%CV) ranged from 0.6 to 13.7%, while accuracy (%bias) ranged from −4.8 to 13.1%. For quality control samples, within- and between-run %CV ranged from 1.5 to 9.9% while %bias ranged from −9.1 to 4.9%. The method requires a sample volume of 0.8 mL and utilizes $^{2}H_{6}$-labeled SCH 201781 as the internal standard. For sample processing, an Isolute C-8 96-well solid phase extraction plate and a Tomtec Quadra 96 sample processor is employed. Separation of the three forms of SCH 201781 is achieved using a 5 μm, 2 mm × 100 mm Asahipak C8 HPLC column and gradient elution. A Sciex API 365 equipped with a turbo ionspray source is used in the selected reaction monitoring mode for detection. The validated method was used to support clinical studies.

Keywords: SCH 201781

1. Introduction

SCH 201781 [*N*-[1-(aminoiminomethyl)-2(*R* and *S*)hydroxy-3(*S*)-piperidinyl]-1,2-dihydro-3-[[(3-methoxyphenyl) sulfonyl]amino]-2-oxo]-1-pyridineacetamide is an orally active thrombin inhibitor currently under study for the treatment of venous and arterial thrombosis.

Venous and arterial thrombosis is one of the leading causes of death in the western world. Currently, heparin is widely used for the treatment of arterial and venous thrombosis [1]. However, the major liabilities to the clinical utility of heparin include plasma protein binding, thrombocytopenia and the dependence of the antithrombotic effects on the plasma cofactors [2]. Direct thrombin inhibitors such as SCH 201781 do not possess the disadvantages of indirect thrombin inhibitors and are equally active against free and clot-bound thrombin [3]. Animal studies have shown consistent evidence of the antithrombotic effects of SCH 201781, suggesting that SCH 201781 is a potent antithrombotic agent with a long duration of action.

To support clinical studies, an automated liquid chromatography–tandem mass spectrometric (LC-MS/MS) method for the determination of SCH 201781 was developed and validated in human plasma.

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2. Experimental

2.1. Materials

Both the test article, SCH 201781, and the internal standard (IS), $^2H_6$-SCH 201781, were synthesized at Schering-Plough Research Institute (SPRI) (Kenilworth, NJ 07033, USA). Both SCH 201781 and the internal standard were stored at 5 °C and protected from light. Blank (analyte-free) heparinized human plasma was purchased from Biological Speciality Corp. (Colmar, PA 18915, USA). The reagents used for extraction and subsequent analysis included methanol (Baxter/Scientific Products, McGaw Park, IL, USA), acetonitrile (JT Baker, Danvers, MA, USA), formic acid and triethylamine (EM Science, Gibbstown, NJ, USA). Water was from a Nanopure-UV water purification system (Barnstead, Dubuque, IA, USA). All reagents were of analytical grade or better and were used prior to their respective expiration dates.

2.2. Stock solutions, calibration standards, and quality control samples

A stock solution of SCH 201781 (200 μg/mL) was prepared in acetonitrile/2 mM ammonium acetate in water (1/1 (v/v)) and used to prepare spiking solutions for the calibration curve standards. A second stock solution (200 μg/mL), prepared from a separate weighing of the same batch of SCH 201781 was chromatographically compared to the first stock solution and shown to agree within 5%. This second stock solution was used to prepare the QC samples. A stock solution of $^2H_6$-SCH 201781 (IS, 200 μg/mL) was also prepared in acetonitrile/2 mM ammonium acetate in water (1/1 (v/v)). This internal standard stock was diluted with acetonitrile/2 mM ammonium acetate/water (1:1:6 (v/v/v)) to prepare and IS working solution at a nominal concentration of 0.626 ng/mL. The stock solutions of the analyte and the IS were mass spectrometrically screened for interfering substances prior to use.

Spiking solutions of SCH 201781 were added to human plasma to achieve the required concentrations of the analyte. A set of eight calibration standards in triplicate ranging from 1.00 to 1000 ng/mL of SCH 201781 were prepared fresh for each analytical run. Quality control (QC) samples were prepared in human plasma pools and stored at −70 °C until analyzed. The following QC pools were prepared: QC at lower limit of quantitation (QC LLOQ, 1 ng/mL), QC low (QC L, 3 ng/mL), QC medium (QC M, 450 ng/mL), and QC high (QC H, 900 ng/mL), and QC dilution (QC D, 1800 ng/mL).

2.3. Extraction procedure

An 800 μL aliquot of each standard or QC sample was pipetted into a separate polypropylene micro-tube. A volume of 50 μL of the IS working solution was added, by the extraction apparatus, to each sample and mixed. The 96-well solid-phase extraction block (Isolute C8, 100 mg, Jones Chromatography, Lakewood, CO 80228, USA) was conditioned with 0.8 mL methanol, then 0.4 mL of water. Following conditioning of the solid-phase extraction block, each sample was applied to a well and washed with 2 mL water, then 1 mL water/acetonitrile (60/40 (v/v)), and air dried at 10 psi for 1 min. Each sample was eluted with 0.5 mL 0.1% triethylamine in methanol into a clean polypropylene elution block and evaporated to dryness under a heated stream of nitrogen set at 40 °C. The resulting residue for each sample was reconstituted with 100 μL of a solution of 0.05% formic acid in water/methanol/acetonitrile (80/10/10 (v/v/v)) and mixed. Each block was then centrifuged at 2000 × *g* for 5 min. A volume of 15 μL of each sample was injected onto the LC-MS/MS system.

2.4. LC-MS/MS conditions

The LC-MS/MS system consisted of two Shimadzu LC-10AD pumps, a Shimadzu SCL-10A A HPLC pump controller, and a PE Sciex API 365 mass spectrometer.

The analytical column used for this assay was a 2 mm × 100 mm, 5 μm Asahipak C8P-50 (Agilent Technologies, Newark, DE 19711). The mobile phase consisted of: mobile phase A, 0.05% formic acid in water; and mobile phase B, 0.05% formic acid in methanol/acetonitrile (1/1 (v/v)). The three forms of the analyte and IS were separated chromatographically using a linear gradient elution (Table 1). The autosampler wash solutions were acetonitrile/water (90/10 (v/v)) and acetonitrile/methanol/0.05% formic acid in water (10/10/80 (v/v/v)). The injection volume was 15 μL.

The API 365 mass spectrometer was operated in positive ion TurboIonSpray mode. The turbo ionspray probe temperature was set at 400 °C with an auxiliary gas flow of 7.5 L/min. The ion spray voltage was set at 4800 V, declustering potential was 52 V, and collision energy was 36 V. The following selected reaction monitoring (SRM) transitions of the respective $[M + H]^+$ ions were used to quantify SCH 201781 in human plasma: SCH 201781 hydrate: *m/z* 497 → *m/z* 321, $^2H_6$-SCH 201781 hydrate: *m/z* 503 → *m/z* 327, SCH 201781 epimers: *m/z* 479 → *m/z* 321, $^2H_6$-SCH 201781 epimers: *m/z* 485 → *m/z* 327. Dwell time for each transition was 250 ms.

The mass axis of the instrument was calibrated by infusion of polypropylene glycol (PPG) 425 in methanol/water (50/50 (v/v)) containing 0.1% formic acid, 0.1% acetonitrile, and 2 mM ammonium acetate, at a flow rate of 10 μL/min. The

Table 1
LC gradient program

Step	Time (min)	Flow rate (μL/min)	%B
1	0.0	200	20
2	5.0	200	30
3	5.3	200	30
4	5.4	200	80
5	5.8	200	80
6	5.9	200	20
7	6.0	200	Stop

sensitivity of the instrument was optimized using an infusion of a 5 μg/mL mixed solution of the analyte and internal standard at 10 μL/min into a flow of 200 μL/min of mobile phase using the initial gradient conditions (30% eluent B). Peak widths were approximately 0.7 ± 0.05 amu at half-height in both single MS and MS/MS modes.

2.5. Software

Sample Control version 1.4 software (PE Sciex Inc., Concord, Ont., Canada) was used for instrument control and data acquisition. MacQuan (v. 1.4) software (PE Sciex Inc., Concord, Ont., Canada) was used for peak integration. Regression was performed using Watson LIMS software (v. 5.3.1.01, PSS, Inc., Wayne, PA 19087).

2.6. Validation and sample analysis study design

Validation was carried out according to the US Food and Drug Administration (FDA) and pharmaceutical industry guidelines [4]. The within- and between-run accuracy and the precision of standards and QCs were assessed with three core validation runs. Precision and accuracy at the LLOQ level was assessed in one of the core validation runs with six QC LLOQ samples. Each core validation run contained three standard curves, QCs at three concentrations (n = 6 at each concentration). Each run also contained a minimum of two blank plasma samples without IS and two with IS (not used in the regression). Six different lots of blank plasma were screened for endogenous interference. Additional validation runs (for stability, recovery, and dilution test) and sample analysis runs contained duplicate standard curves, QCs at three concentrations (n = 3 at each concentration), and a minimum of four blanks (two without IS and two with IS).

3. Results and discussion

3.1. Isomers and hydrate

In aqueous solution, SCH 201781 exists in three forms: a piperidine ring-opened hydrate and two ring-closed epimers, A and B (Fig. 1). The three forms of SCH 201781 were separated chromatographically in this assay (Fig. 4). The full-scan and product ion mass spectra were obtained using LC-MS and LC-MS/MS, respectively, in the positive ion mode. The full-scan single MS spectrum of the hydrate form of SCH 201781 showed an abundant protonated molecular ion at m/z = 497 while the full-scan single MS spectra of the two epimeric forms of SCH 201781, A and B, showed abundant protonated molecular ions at m/z = 479. The product ion mass spectra of the hydrate and epimers are shown in Fig. 3. All product ion mass spectra exhibit a major fragment ion at m/z 321, a result of fragmentation at the acetamide bond to form an acylium ion. Therefore, transitions m/z 497 → m/z 321 and m/z 479 → m/z 321 were used for the hydrate and epimers respectively.

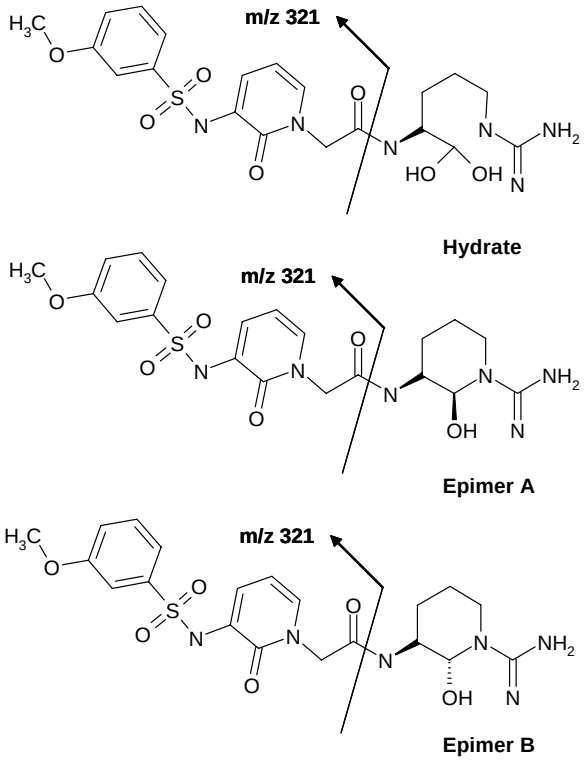

Fig. 1. Three forms of SCH 201781 structures in aqueous solution: a piperidine ring-opened hydrate and two ring-closed epimers, A and B.

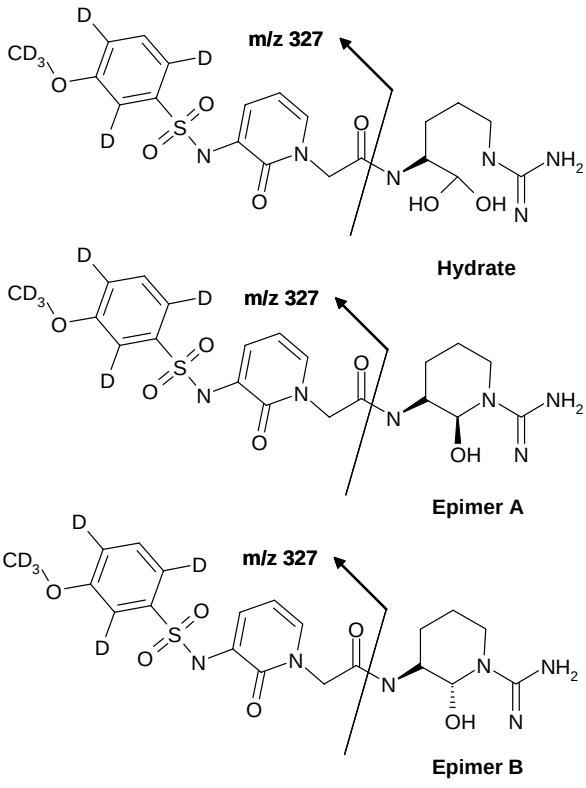

Fig. 2. Three forms of $^{2}H_{6}$-SCH 201781 structures in aqueous solution: a piperidine ring-opened hydrate and two ring-closed epimers, A and B.

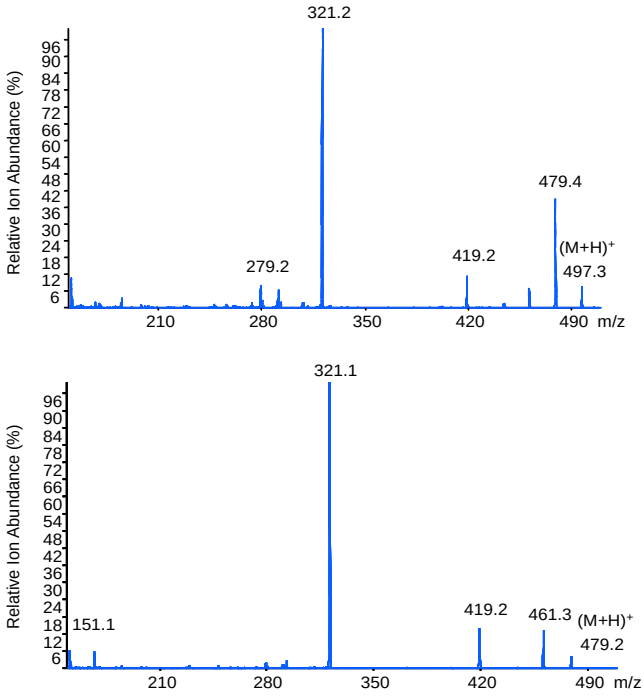

Fig. 3. Full-scan LC-MS/MS product ion spectrum of SCH 201781. Top: hydrate (products of *m/z* 497); bottom: epimers A and B (product of *m/z* 479).

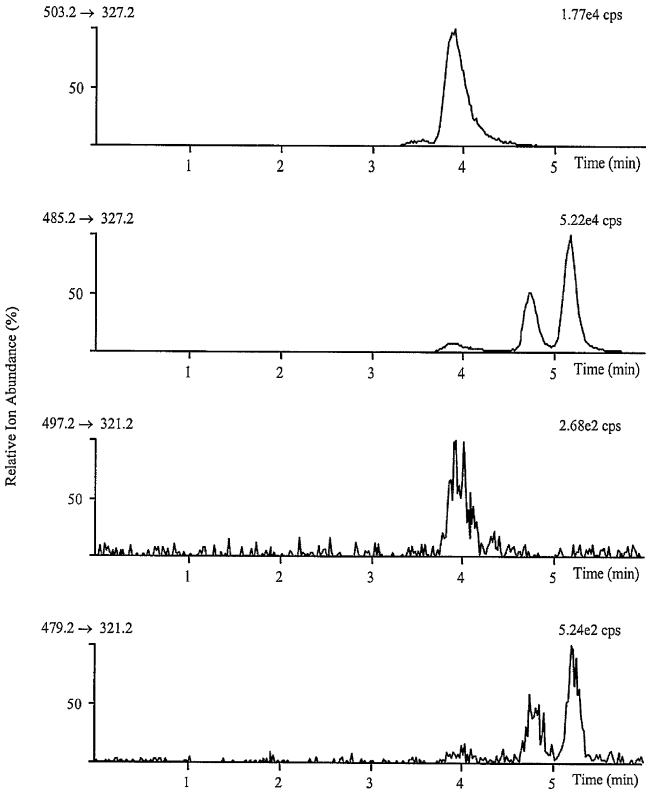

Fig. 4. Representative SRM chromatograms for a calibration curve standard at LLOQ level (1.0 ng/mL).

The internal standard $^{2}H_6$-SCH 201781, has six deuteriums incorporated at the methoxy methyl and the 2, 4, and 6 positions of the methoxy phenyl ring (Fig. 2). $^{2}H_6$-SCH 201781 also exists in aqueous solution in three forms: a ring-opened hydrate and two ring-closed epimers. Similar to SCH 201781, the predominant protonated molecular ions for $^{2}H_6$-SCH 201781 are: *m/z* 503 for the hydrate form and *m/z* 485 for the epimers, all with a major fragment ion at *m/z* 327 (data not shown). Therefore, transitions *m/z* 503 → *m/z* 321 and *m/z* 485 → *m/z* 321 were used for the IS hydrate and IS epimers, respectively.

3.2. Separation, sensitivity, and selectivity

Although only total SCH 201781 concentrations were determined in this assay, it was decided that the three forms (two epimers and one hydrate) of SCH 201781 should be separated chromatographically in case there was a need to quantify each of the forms separately. This was achieved using an Asahipak C8P-50 column and a gradient elution. The co-eluting forms of SCH 201781 and the IS were separated by mass using precursor to product ion fragmentation (tandem MS/MS).

A representative reconstructed ion chromatogram of SCH 201781 standard at LLOQ level is shown in Fig. 4 with adequate signal to noise level. Accuracy (%bias) and precision (%CV) at the LLOQ level were determined in one of the validation runs in which six replicates of QC samples at 1 ng/mL were processed and analyzed along with other QC samples and standards. The %bias was 1.2% and %CV was 2.1%.

Selectivity of the assay was demonstrated by screening six lots of blank plasma. All six lots screened were free of significant (>10% of the LOQ) interference from endogenous components or other sources at the retention time of the analyte and IS. A chromatogram of an extracted blank human plasma sample is shown in Fig. 5.

3.3. Regression, accuracy, and precision

Following peak area integration, the results tables from MacQuan were saved as three text files (representing the ring-opened hydrate and two ring-closed epimeric forms of SCH 201781, A and B) for each run. These text files were opened in Excel 97; a macro was used to sum the areas from the three files for both the analyte and IS. The resulting text file was then saved and uploaded to a file server where regression was performed using Watson LIMS software (v. 5.3.1.01, PSS Inc., Wayne, PA 19087). A linear equation with a weighting of 1/concentration^2 was used for each calibration curve (i.e. $y = mx + b$, where y is the peak area ratio of SCH 201781 to IS, x the concentration of SCH 201781, and m and b are curve parameters). The simplest mathematical model that adequately fit the concentration–response relationship was used. The concentration of SCH 201781 in each plasma sample was determined by inverse prediction from the calibration curve equation.

Table 2
Precision and accuracy for calibration curve standards of SCH 201781

Run #	Statistics	STD 1 1.00 ng/mL	STD 2 2.00 ng/mL	STD 3 5.00 ng/mL	STD 4 25.0 ng/mL	STD 5 100 ng/mL	STD 6 500 ng/mL	STD 7 750 ng/mL	STD 8 1000 ng/mL
1	Mean	1.02	1.93	4.97	24.1	102.8	517.6	738.8	1011
	%CV	3.6	12.5	1.7	11.1	4.0	1.9	5.8	5.7
	%bias	2.1	−3.7	−0.6	−3.8	2.8	3.5	−1.5	1.1
2	Mean	1.01	1.97	4.89	24.1	95.5	509.5	714.4	1131
	%CV	8.1	6.2	7.5	3.8	6.9	0.6	0.7	5.7
	%bias	1.4	−1.6	−2.2	−3.5	−4.5	1.9	−4.8	13.1
3	Mean	1.02	1.96	4.79	25.4	101	500	747	1022
	%CV	13.7	4.6	8.1	5.3	4.5	3.3	5.0	3.2
	%bias	1.6	−1.8	−4.2	1.7	0.9	−0.88	−0.4	2.2
Between-run	Mean	1.02	1.95	4.88	24.5	99.7	509	733	1055
	%CV	8.2	7.4	5.8	6.9	5.6	2.5	4.4	7.00
	%bias	1.7	−2.3	−2.4	−1.9	−0.26	1.8	−2.2	5.5

Data calculated with unrounded numbers.

For calibration curve standards, within- and between-run %CV ranged from 0.6 to 13.7%, while %bias ranged from −4.8 to 13.1% (Table 2). For quality control samples, within- and between-run %CV ranged from 1.5 to 9.9% while %bias ranged from −9.1 to 4.9% (Table 3). Coefficients of determination (r^2) for validation runs were 0.993 or greater.

3.4. Recovery

Recovery of SCH 201781 from human plasma was determined by comparison of the peak areas of the low, medium, and high QCs (pre-extract spike) to those of the respective post-extract spiked QC samples. Pre- and post-extract spiked QC samples were compared to neat solutions to determine possible ion suppression or matrix effect (Table 4). Mean observed recovery of SCH 201781 from pre-extract spiked samples in comparison to post-extract spiked samples ranged from 38.3 to 52.4%. Mean observed recovery of SCH 201781 from pre-extract spiked samples in comparison to neat solutions of analyte dissolved in solvent ranged from 32.1 to 40.6%. Comparison of these data with the post-extract spiked

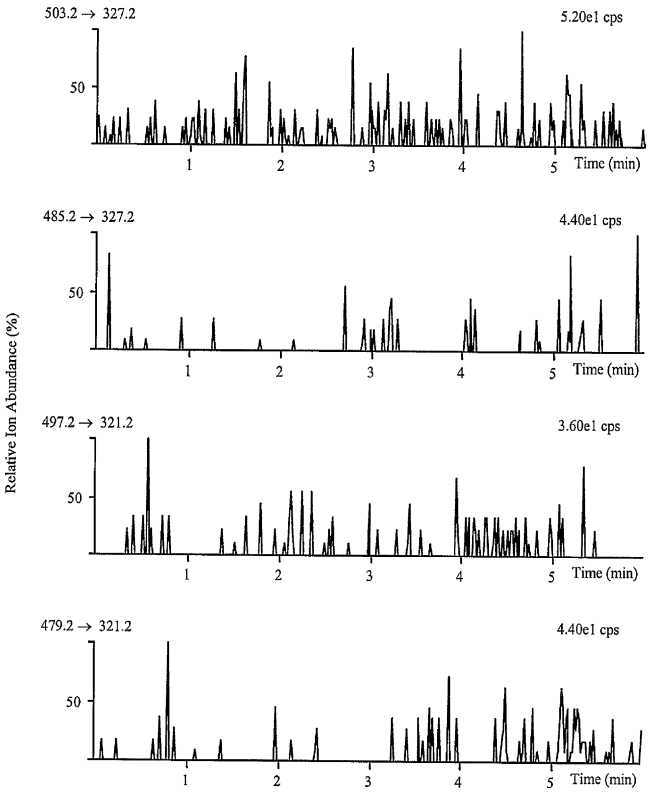

Fig. 5. Representative SRM chromatograms of an extracted blank human plasma sample.

Table 3
Precision and accuracy for quality control samples

Run #	Statistics	QC 1 3.00 ng/mL	QC 2 450 ng/mL	QC 3 900 ng/mL
1	Mean	3.15	409	819
	%CV	9.9	1.6	6.0
	n	6	6	6
	%bias	4.9	−9.1	−9.0
2	Mean	3.06	458	893
	%CV	4.2	1.5	5.1
	n	6	6	6
	%bias	2.0	1.9	−0.7
3	Mean	3.14	449	905
	%CV	5.7	5.2	6.1
	n	6	6	6
	%bias	4.6	−0.3	0.5
Between-run	Mean	3.12	440	876
	%CV	6.7	5.8	6.9
	n	18	18	18
	%bias	3.9	−2.1	−2.7

Data calculated with unrounded numbers.

Table 4
Recovery for SCH 201781

QC	Pre-extract/ post-extract	Pre-extract/neat	Post-extract/neat
QC low	54.0	42.8	79.3
QC median	49.8	40.3	81.2
QC high	44.0	36.3	82.5

samples indicates minimal ion suppression or matrix effect of analyte signal.

3.5. Dilution integrity

The effect of dilution on the analysis of SCH 201781 was determined by partial volume analysis. For this purpose, a dilution QC sample (QC D) was prepared containing SCH 201781 in human plasma at approximately twice the QC high concentration. Six replicates of QC D (1800 ng/mL) were diluted 10-fold and analyzed. Precision and accuracy for QC D were 4.0 and −3.1%, respectively.

3.6. Stability

Stability of SCH 201871 under various storage and process conditions was evaluated by comparing the mean concentrations of stability QC samples (low, median, and high, n = 6) to their respective Time 0 concentrations. SCH 201781 was determined to be stable if both the deviation from Time 0 (%bias) and precision (%CV) of those stability QC samples are not >15%. Using these criteria, SCH 201781 was determined to be stable in human plasma at room temperature under yellow light for up to 24 h, frozen at −70 °C for up to 18 weeks, and after three freeze–thaw cycles. Extracted SCH 201781 was stable in reconstitution solution at room temperature for up to 32 h.

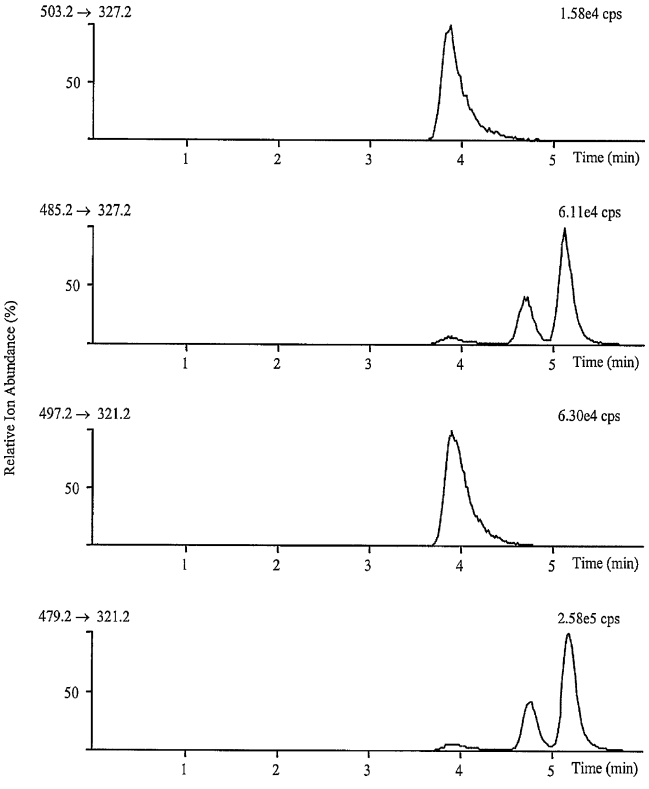

Fig. 6. Representative SRM chromatograms from a human plasma study sample.

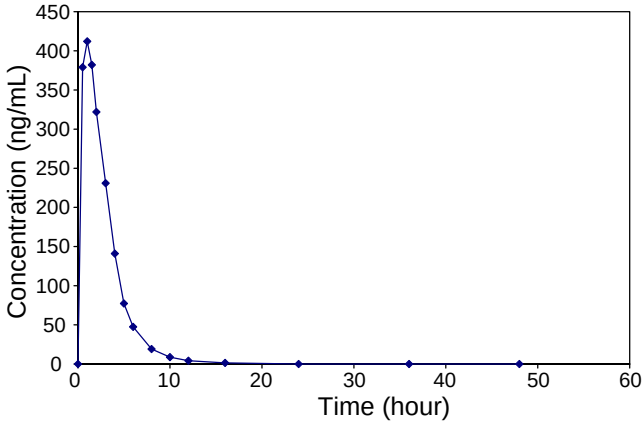

Fig. 7. A typical concentration-time profile of SCH 201781 in human plasma from a study subject receiving 5 mg/kg dose.

4. Application of the method

The validated LC-MS/MS method was used to support clinical studies. The first clinical study supported using this validated method was a rising single dose study for the safety and tolerability of SCH 201781 in human. The study involved six dose groups (1, 2.5, 5, 10, 20, and 25 mg/kg) with six subjects per group. SRM chromatograms of a 5 mg/kg dose, 3 h sample from that study is shown in Fig. 6. Concentration–time profile of SCH 201781 from a subject receiving 5 mg/kg dose is presented in Fig. 7. Data from this study indicated that SCH 201781 was rapidly absorbed and eliminated in humans with a relatively short half-life.

5. Conclusion

An automated 96-well solid-phase extraction LC-MS/MS method was developed and validated for the quantitation of SCH 201781 in human plasma. The calibration curves showed goodness of fit over the concentration range of 1.0–1000 ng/mL using a linear regression with 1/concentration^2 weighting. Within- and between-run precision and accuracy for calibration standards and QCs met FDA suggested acceptance criteria. SCH 201781 was stable in human plasma under the storage and test conditions used for this assay. These results indicate the method to be sensitive, specific, accurate, and reproducible for the determination of SCH 201781 concentrations in human plasma samples.

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