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Quantifying tetrapeptide SS-20 in rat plasma using hydrophilic interaction liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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ABSTRACT

Quantitative analysis of peptides in biological matrices remains a challenging task. This is due to the low dosage and the complexity of both the matrix and the analytical characteristics of peptides. SS-20 is a tetrapeptide compound developed for the treatment of Parkinson's disease. To investigate the pharmacokinetics of SS-20, a sensitive and rapid liquid chromatography coupled with mass spectrometry method was developed and validated. An aliquot of 50 μL plasma sample was extracted via solid phase extraction. The extracts were separated using a hydrophilic interaction liquid chromatography column, and were then detected with a triple quadrupole mass spectrometer using electrospray ionization in positive-ion mode and selected reaction monitoring. The use of a deuterium-labeled internal standard provided acceptable accuracy, precision, and matrix effect. The lower limit of quantification was 0.30 ng/mL. The linear range of the method was from 0.30 to 1000 ng/mL. The intraday and interday precisions were lower than 10.2% in terms of relative standard deviation, and the accuracy was within ±2.1% in terms of relative error. The validated LC–MS/MS method was successfully applied to a pharmacokinetic study of SS-20 following an intravenous or subcutaneous injection administration of 1.0 mg/kg to Sprague-Dawley rats.

1. Introduction

Biomolecules such as therapeutic peptide and proteins have recently received prominence in the pharmaceutical industry because they tend to be less toxic and have less drug–drug interaction susceptibilities than small molecule drugs. Therapeutic proteins currently represent over 25% of newly approved drugs on the European market, and half of all drugs in the development phase [1]. The pharmacokinetics and pharmacokinetic/pharmacodynamic relationships affect each stage of the development of peptides. To develop these biomolecules further, quantification techniques for these analytes in the biological matrix are greatly needed. However, the main challenge in developing these analytes is quantifying them in biological environments. Most of the conventional peptide quantification is by immunoassays. Immunoassays can provide better limits of quantification. However, immunoassays often quantify the total amount of structurally related peptides, limiting the accuracy of the analysis. Although liquid chromatography with tandem mass spectrometry (LC–MS/MS) is the method of choice for sensitive and selective detection of compounds in complex biological matrices, it still has some drawbacks for peptide quantification. Peptides are composed of amino acids that are similar to endogenous peptides, making the separation of these peptides from the biological matrix difficult. The separation of the peptide from interfering matrix components prior to detection by sample pre-treatment and chromatography separation are extremely important. Peptides are easily absorbed onto the container surface, which becomes another challenge for developing an appropriate method for peptide quantification. Solid phase extraction or precipitation is the most efficient sample preparation for peptide quantification in biological matrix, which provide sufficient deproteinization, desalting, and clean-up [2,3]. Reversed phase liquid chromatography (RP-LC) [2,3] and ion-exchange chromatography (IEC) [3] are among the widely used stationary phases for separating peptides from interfering components. Hydrophilic interaction chromatography (HILIC) uses polar stationary phases combined with aqueous mobile phases, which provides better solubility for peptides. The advantage of separating the polar compounds makes the HILIC column popular.

Yoshida reviewed peptide separations using HILIC [4]. Weng reviewed recent advances in application of HILIC for quantitative bioanalysis [5]. Thus far, only few quantitative HILIC assays have been performed for peptides in biological matrices. In the current study, SS-20 was used as a model peptide in developing a

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relatively fast and sensitive quantification method for peptides by HILIC column separation.

SS-20 (Phe-D-Arg-Phe-Lys–NH_2), an SS (*Szeto–Schiller*) peptide, is a drug candidate for the treatment of Parkinson's disease [6]. The structural motif of SS peptides is centered on alternating aromatic residues and basic amino acids (aromatic–cationic peptides) [7]. SS-20 is a synthetic tetrapeptide, which is potent (nM) in preventing 1-methyl-4-phenylpyridinium (MPP^+)-induced cell death in cultured dopamine cells (SN4741) [6]. SS-20 is structurally similar to DALDA (Tyr-D-Arg-Phe-Lys–NH_2), which is a mu opioid-receptor agonist. Grigoriants et al. [8] reported a cf-LSIMS method for DALDA quantification in ovine plasma, with a limit of detection of 4.00 ng/mL and a total run time of more than 20 min. Wan et al. [9] developed an online liquid chromatography coupled with a quadrupole time-of-flight mass spectrometry for [Dmt^1] DALDA quantification in ovine plasma using C_{18} column separation. The lower limit of quantification (LLOQ) was 4.00 ng/mL for a 200-μL ovine plasma, and the total run time was more than 14 min. Wan et al. [10] later developed an liquid chromatography quadrupole ion-trap mass spectrometer for [Dmt^1] DALDA quantification in ovine plasma with an LLOQ of 16.0 ng/mL and a run time of more than 9 min. To investigate its pharmacokinetics, a reliable and fast analytical method with adequate sensitivity is necessary.

The aim of this study is to develop a rapid and sensitive LC–MS/MS method for SS-20 quantification in rat plasma. One of the SS peptides has been studied in the clinical research phase. A Waters Atlantis HILIC column was used to separate SS-20 from the biological matrix. The present method is the first attempt in SS peptide quantification by HILIC column separation. This method exhibited excellent performance in terms of high sensitivity, wide linear concentration range, and short chromatographic run time. After full validation, the method was successfully applied to a pharmacokinetic study of SS-20 after intravenous or subcutaneous injection of 1.0 mg/kg of SS-20 into rats.

2. Experimental

2.1. Reagents and materials

SS-20 (85.4%, peptide content) and d_{10}-SS-20 (63.9%, peptide content) were obtained from Stealth Peptides International (Shanghai) Inc. Methanol and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). HPLC grade formic acid and acetic acid were purchased from Tedia (Fairfield, OH, USA). Heparinized blank (drug-free) rat plasma was supplied by the Laboratory Animal Center of our institute. Millipore Milli-Q gradient purified water (Molsheim, France) was used throughout the study. An Oasis® HLB cartridge (30 mg, 1 mL) was purchased from Waters Corp. (Milford, MA, USA).

2.2. Instrument

An Agilent 1100 liquid chromatography system equipped with a G1311A quaternary pump, a G1322A vacuum degasser, a G1316A thermostatted column oven, and a G1367A autosampler (Agilent, Waldbronn, Germany) was used. Mass spectrometric detection was performed on a Thermo Finnigan TSQ Quantum Ultra triple quadrupole instrument (San Jose, CA, USA) in selective reaction monitoring (SRM) mode. An electrospray ionization (ESI) interface in positive ionization mode was used. The Agilent ChemStation and Finnigan Xcalibur software packages were used to control the LC–MS/MS, as well as data acquisition and processing.

2.3. Chromatographic conditions

Chromatographic separation of the prepared samples was achieved at 25 °C using Atlantis® HILIC Silica column (150 mm × 2.1 mm I.D., 3 μm, Waters, Ireland) with a SecurityGuard C_{18} column (4 mm × 3.0 mm I.D., 5 μm, Phenomenex, Torrance, CA, USA). A mobile phase of methanol–water–formic acid (70:30:0.1, v/v/v) was employed. An initial flow of 0.5 mL/min was maintained for 1.8 min, which was then increased to 0.65 mL/min to wash the column (divert to waste) over the next 1.2 min.

The chromatographic run time of each sample was within 3.0 min. A post-column divert value was used to direct the HPLC eluate to the ionization source in the first 2 min of the chromatographic run, and then to the waste container.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in positive ion mode using an ESI source for SS-20 and IS. The tuning parameters were optimized for SS-20 and IS by infusing a solution containing 1000 ng/mL of the analyte and IS at a flow rate of 20 μL/min into the mobile phase (0.45 mL/min) using a post-column "T" connection. The sheath gas, auxiliary gas, and ion sweep gas (nitrogen) were set at 35, 5, and 2 Arb, respectively. The spray voltage was set at 4200 V, and the interface capillary temperature was maintained at 350 °C. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of 1.2 mTorr. Quantification was performed using selected reaction monitoring (SRM), which monitors the transitions m/z 298.6 → m/z (84.0 + 120.0) for SS-20 and m/z 303.5 → m/z (84.0 + 125.0) for deuterated IS, with a dwell time of 200 ms per transition. An optimized collision energy of 30 eV was used for the analyte and IS. The mass spectrometer was operated at the unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3.

2.5. Preparation of standard and quality control (QC) samples

A standard stock solution of SS-20 was prepared by dissolving the accurately weighed reference compound in methanol to give a final concentration of 1083 μg/mL (calculated as peptide content). The solution was then serially diluted with methanol to give working solutions at concentrations of 0.30, 1.00, 5.00, 20.0, 100, 300, and 1000 ng/mL. Calibration curves were prepared by spiking 50 μL of the appropriate standard solution into 50 μL of the blank rat plasma. The quality control (QC) solutions were similarly prepared at concentrations of 0.80, 100, and 900 ng/mL in rat plasma by a separate weighing of the reference compound. A 100 ng/mL solution of IS was also prepared by diluting the 30 μg/mL (calculated as peptide content) stock solution of deuterated SS-20 with methanol. All of the solutions were stored at 4 °C and were brought to room temperature before use. QC samples were aliquoted into 1.5 mL polypropylene vials and stored at −20 °C until the analysis was performed.

To minimize nonspecific binding, polypropylene tubes were used throughout the whole study.

2.6. Sample preparation

Frozen plasma samples were thawed by transferring it to an ice/water bath prior to preparation. All of the preparations were done at 4 °C according to the short-term stability data. After vortexing, a 50 μL aliquot of the IS solution (d_{10}-SS-20, 100 ng/mL), 50 μL methanol, and 400 μL water were added to 50 μL of the plasma sample. After vortex mixing for 1 min and centrifugation at 11,300 × g for 5 min at 4 °C, the supernatant was then loaded onto Oasis® HLB solid phase extraction tubes that were sequentially pretreated with 2 × 1 mL of methanol and 2 × 1 mL of water. After loading the plasma sample, the cartridge was washed with 1 mL of water, and the peptide-enriched fraction was eluted with 2 × 1 mL of methanol containing 1% acetic acid. The eluate was evaporated to

dryness at 40 °C under a gentle stream of nitrogen, and the residue was reconstituted by adding 100 μL of the mobile phase. A 20 μL aliquot was injected onto the LC–MS/MS system for analysis.

2.7. Method validation

The selectivity of the method was evaluated by analyzing six blank plasma samples and six spiked plasma samples at the LLOQ level from six different rats. The peak areas of the endogenous compounds co-eluted with the analytes should be less than 20% of the peak area of the LLOQ standard according to international guidelines [11]. The internal standard was present in the bioanalytical assay to compensate for the extraction variability in the LC–MS/MS analysis. A highly variable internal standard can be an indication of an uncontrolled process during sample analysis [12].

Linearity was assayed by assaying the calibration curves in rat plasma in duplicates in three separate runs. The curves were then fitted by a linearly weighed ($1/x^2$) least squares regression method through the measurement of the peak area ratio of the analyte to IS.

To evaluate the precision and accuracy of the method, QC samples at three concentrations (0.80, 100, and 900 ng/mL) were analyzed in six replicates on three validation days. The precision was expressed as RSD, and the assay accuracy was expressed as RE, i.e., (observed concentration − nominal concentration)/(nominal concentration) × 100%. The intraday and interday precisions were required to be below 15%, and the accuracy to be within ±15% [11].

Dilution of the biological matrix is required if the studied sample concentration were higher than the upper limit of quantification. Dilution validity experiments were carried out by a 10-fold dilution of the plasma samples with blank plasma for six replicates. The acceptable precision and accuracy were required to be within ±15%.

The post-column infusion method was utilized to measure qualitatively the matrix effect. To quantitatively evaluate the matrix effect, the matrix factor was measured as defined by the ratio (analyte peak area ratio of the analyte to IS in presence of plasma matrix)/(analyte peak area ratio of the analyte to IS in absence of plasma matrix), and was expressed as the percent response relative to the neat solution. In our experiment, the matrix effect was determined at two concentrations (0.80 and 100 ng/mL). The intersubject variability of the matrix effect should be less than 15% [13].

The extraction recoveries of SS-20 were estimated by comparing the peak areas of the analytes in the extracted QC samples ($n = 6$) with those obtained from the extracted blank plasma samples post-spiked with corresponding neat solutions in six replicates. The extraction recoveries of IS were determined in a similar way using the QC samples at high concentration as a reference.

The stabilities of SS-20 in rat plasma were evaluated by analyzing the replicates ($n = 3$) of plasma samples at 0.80 and 900 ng/mL for SS-20, which were exposed to different conditions (time and temperature). These results were compared with those obtained from freshly prepared plasma samples. The analyte was considered stable in the biological matrix when 85–115% of the initial concentrations were found. The short-term stability was determined after exposing the spiked samples to room temperature for 1 h, and to +4 °C for 1 h and 24 h. The freeze/thaw cycles stability was evaluated after one or three complete freeze/thaw (−70 to 4 °C) cycles in consecutive days. The stability of the extracted samples was assessed by re-injecting the intra-assay standards after 24 h of storage at room temperature. Long-term stability was assessed after storage of the standard spiked plasma samples at −70 °C for 11 days. The stabilities of the standard solution were also investigated at 4 °C for 5 days, and for 6 h at room temperature.

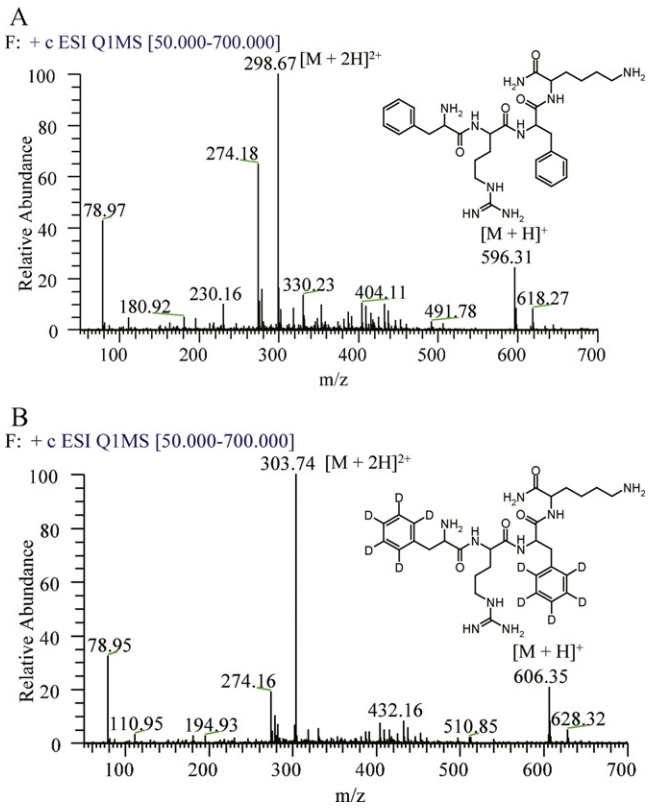

Fig. 1. Full-scan mass spectra of SS-20 (A) and d_{10}-SS-20 (B).

2.8. Application to a pharmacokinetic study

The Sprague-Dawley rats (250 ± 20 g) used in the pharmacokinetic study of SS-20 were provided by the Laboratory Animal Center of our institute. Rat chow and water were given *ad libitum*. SS-20 dissolved in physiological saline was administered to rats by intraveneous or subcutaneous injection (5 mL/kg) at a dose of 1.0 mg/kg. Blood samples of about 0.2 mL each were collected into heparinized tubes (on ice) from each rat by puncturing the retro-orbital sinus. This was performed according to a predetermined time schedule of 0.08, 0.25, 0.75, 2.0, 4.0, 6.0, 8.0, 12.0, and 24.0 h. Plasma was immediately obtained from each sample by centrifugation at 11,300 × *g* (4 °C) for 5 min, and stored at −70 °C until analysis.

3. Results and discussion

3.1. Mass spectrometric condition

SS-20 contains lysine (Lys) and arginine (Arg) residues in its structure. The presence of these two basic amino acids resulted in a favorable sensitivity for SS-20 in the positive ESI ionization mode. In the positive ESI, both SS-20 and IS, doubly protonated molecules $[M+2H]^{2+}$ at m/z 298.6 and m/z 303.6, were the most abundant compared with their single charged molecular ions in Q1 full-scan mass spectra (Fig. 1). As a result, the $[M+2H]^{2+}$ of each compound was selected as the precursor ion for generating the specific peptide segment information. Fig. 2 shows the product ion spectra of SS-20 and IS, which have the same type of product ions: a_1, b_2, z_1, and b_2–NH_3. To improve the sensitivity of SS-20 and IS, the transitions of m/z 298.6 → 84.0 and m/z 298.6 → 120.0 were both chosen for SS-20, m/z 303.5 → 84.0 and m/z 303.5 → 125.0 for IS.

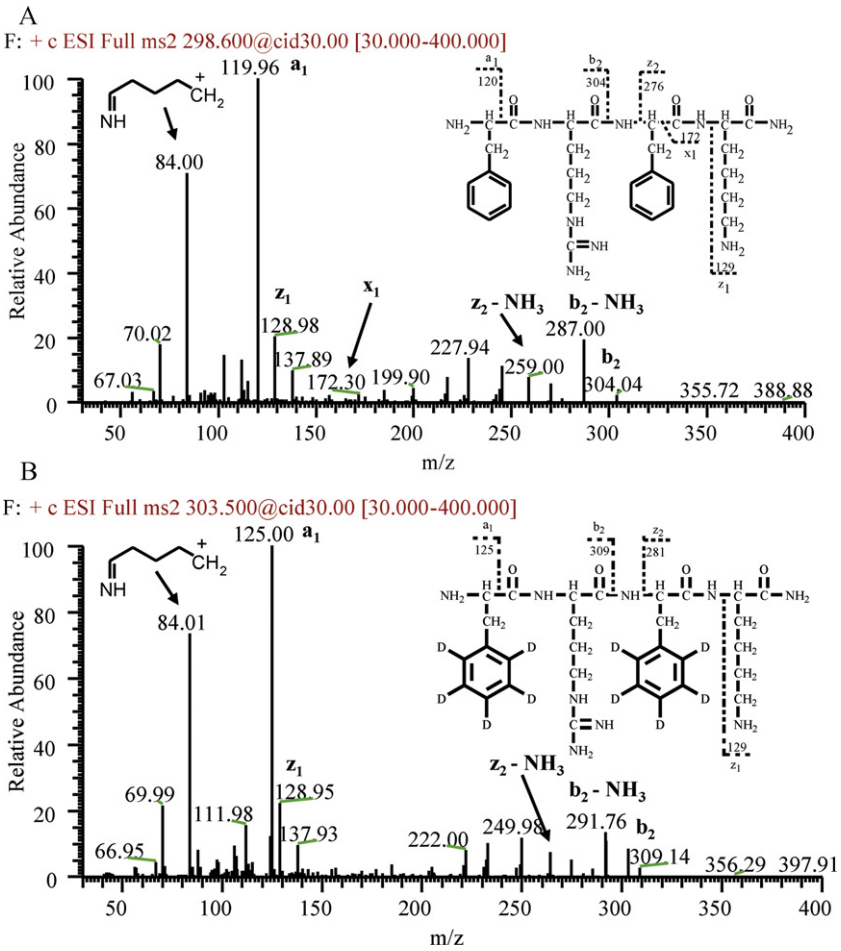

Fig. 2. Product ion mass spectra of $[M+2H]^{2+}$ ion of SS-20 (A) and d_{10}-SS-20 (B).

3.2. Chromatographic condition

For peptide analysis, the chromatographic separation is still a challenging task. Separating the peptide from the endogenous compounds on chromatography, e.g., phospholipid, is difficult due to the polarity of the peptide. The co-elution of endogenous compounds easily induces the matrix effect. In optimizing the LC system, several brand chromatographic columns were tested, such as Zorbax Eclipse XDB-C_8, Phenomenex Jupiter C_4, and Waters XTerra C_{18} column. However, high proportion water phase or long carbon chain acidic modifiers had to be included in mobile phase system. Then the ESI-MS response of SS-20 was decreased by the high proportion water phase. Wan et al. [9,10] used a home-packed C_{18} column for [Dmt^1] DALDA separation under an isocratic chromatographic system with a run time of 15 and 10 min, respectively. HILIC is characterized by the use of a hydrophobic organic mobile phase and a hydrophilic stationary phase. The order of elution is reversed relative to reversed-phase chromatography (RP), with the hydrophilic compounds being retained longer than the hydrophobic ones. Therefore, HILIC can simply be seen as a form of normal phase chromatography. Recently, HILIC has been used widely in the separation of polar compounds. In the experiment, different types of HILIC columns from different suppliers, such as Phenomenex Luna HILIC, Agela HILIC, and Waters Atlantis® HILIC Silica, were also tested. Among the HILIC columns tested, the best performance was achieved with Waters Atlantis® HILIC Silica (150 mm × 2.1 mm I.D., 3 μm, Waters, Ireland).

The best results were obtained with a mobile phase consisting of methanol–water (70:30, v/v), and a great improvement in the peak shape and retention time was achieved when 0.1% formic acid was added. SS-20 is a *Szeto–Schiller* peptide, and many endogenous proteins in the plasma easily interfere with SS-20 quantification in biological samples. Therefore, chromatographic retention and separation are important. To avoid the interference peaks during the analysis, the flow was changed as follows: the initial flow of 0.5 mL/min was maintained for 1.8 min, and was then increased to 0.65 mL/min for column washing (divert to waste) over the next 1.2 min. As a result, the analytical run time of each sample was about 3 min.

3.3. Sample preparation and adsorption issue

Currently, the employed techniques for peptide recovery from biological matrices are solid phase extraction (SPE), protein precipitation (PPT), liquid–liquid extraction (LLE), immunoaffinity purification, and ultrafiltration [3]. In the current study, different sample preparation procedures were attempted. LLE gave a low SS-20 recovery. After PPT with acetonitrile, a significant chromatographic interference and a great extent of ionization suppression were observed. SPE can concentrate the target analyte from a liquid matrix onto a suitable solid phase, and can provide an efficient means of analyte enrichment. Further experiments showed that SPE gave consistent recovery rates with minimum matrix interference, and could therefore provide a robust assay. Different SPE cartridges were investigated according to extraction recovery, including Strata-E, Strata-ph, Orochem C_{18}, Strata-X, and Oasis® HLB cartridges. Among them, the Oasis HLB cartridge was the most satisfactory SPE cartridge. The different loads, washes, and elution

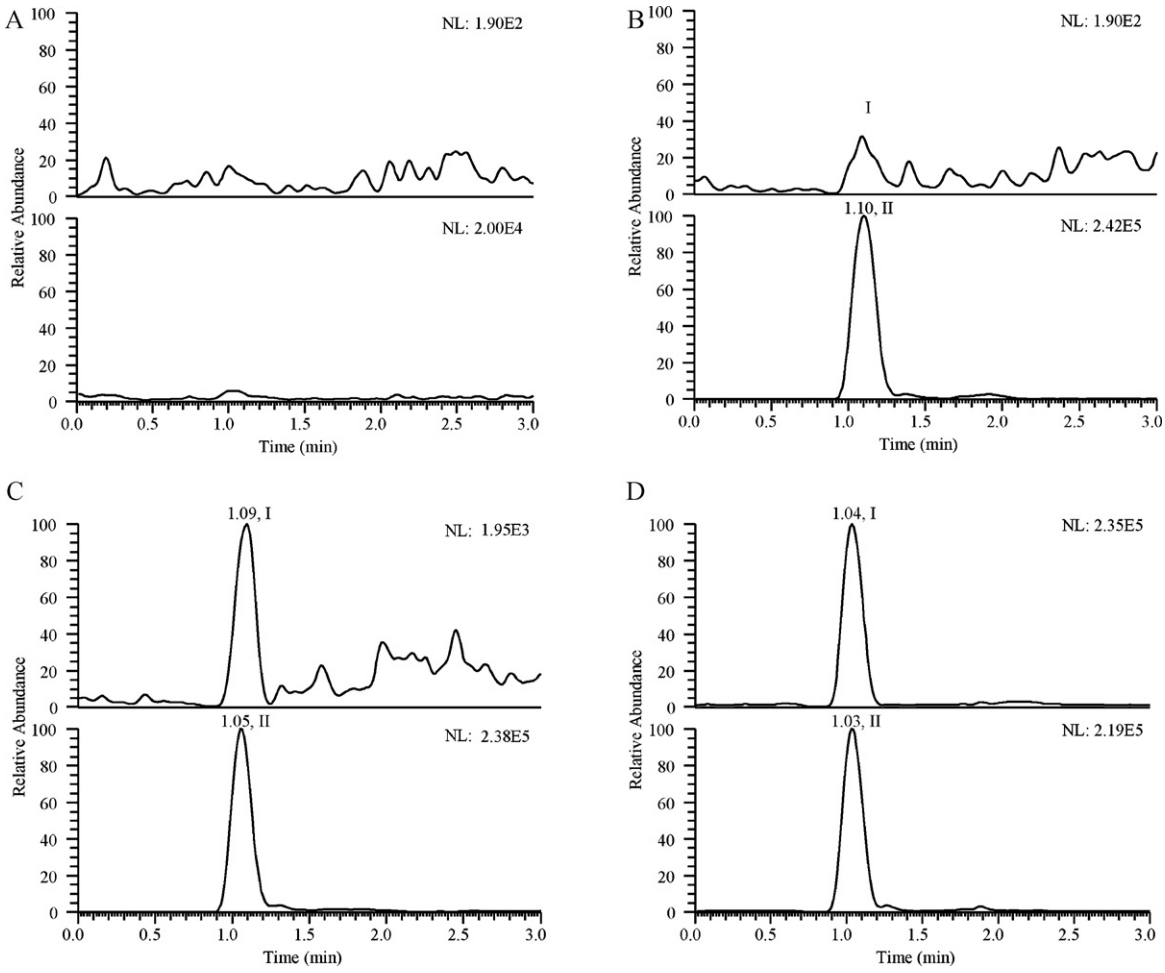

Fig. 3. Representative SRM chromatograms for SS-20 (I) and d_{10}-SS-20 (IS, II) in rat plasma: (A) a blank rat plasma sample; (B) a blank plasma sample spiked with d_{10}-SS-20 (IS) at 100 ng/mL; (C) a blank plasma sample spiked with SS-20 at 0.300 ng/mL and d_{10}-SS-20 (IS) at 100 ng/mL; (D) a rat plasma sample obtained at 6.0 h after an intravenous injection of 1.0 mg/kg SS-20.

solvents were then further optimized. Based on the optimization, 1% acetic acid in methanol (2 × 1 mL) was used as the elution solvent, which resulted in the highest recovery rates for SS-20 and IS.

Some peptides [14–18] are adsorbed onto container surfaces, which can lead to poor responses and non-linear correlations. During the initial study, SS-20 showed strong adsorption onto glass surfaces. Consequently, different plastic tube materials from different sources were evaluated to minimize the adsorption. According to the trial, the polypropylene tubes showed minimal SS-20 adsorption. Polypropylene tubes were hence used for the sample preparation during the whole study.

3.4. Method validation

3.4.1. Assay selectivity and matrix effect

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma samples at the LLOQ levels. Fig. 3 shows the typical chromatograms of a blank rat plasma, blank plasma sample spiked with SS-20 at the LLOQ and IS, and a plasma sample obtained at 6.0 h after intravenous injection of 1.0 mg/kg SS-20 into an SD rat. No significant interference from the endogenous substances was observed at the retention times of SS-20 and IS. The typical retention times for both SS-20 and IS were at 1.05 min. The corresponding capacity factor for both SS-20 and IS were 0.8.

As for the matrix effect, the absolute matrix effects for SS-20 in six different lots of rat plasma at concentrations of 0.80 and 100 ng/mL were 82.7% and 81.3%, respectively. The relative matrix effect was 5.7% and 3.1%, respectively. The absolute and relative matrix effects for IS (100 ng/mL in plasma) were 82.4% and 9.6%, respectively. Although there was an extent to the matrix inhibition for SS-20 in the present condition, the accurate determination of SS-20 in rat plasma was not influenced.

3.4.2. Linearity of calibration curve and lower limit of quantification

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 0.30–1000 ng/mL for SS-20 in rat plasma. A typical equation of the calibration curve on a validation day was as follows: $y = 1.52 \times 10^{-2}x + 2.66 \times 10^{-3}$ ($r^2 = 0.9927$), where y represents the peak area ratio of the analyte to the IS, and x represents the plasma concentration of SS-20. Good linearity was obtained in this concentration range.

The LLOQ was established at 0.30 ng/mL for SS-20. The precision and accuracy corresponding to the LLOQ are shown in Table 1. Under the present LLOQ of 0.30 ng/mL, the plasma concentration of SS-20 could be determined for up to 24 h after the intravenous administration of 1.0 mg/kg SS-20 into SD rats, which is sensitive enough to investigate the pharmacokinetic behavior of SS-20.

Table 1
Precision and accuracy of the LC–MS/MS method to determine SS-20 in rat plasma (in three consecutive days, six replicates for each day).

Concentration (ng/mL)		RSD (%)		RE (%)
Added	Found	Intra-day	Inter-day	
0.30 (LLOQ)	0.30 ± 0.029	10.2	1.7	−0.02
0.80 (L)	0.78 ± 0.059	8.0	3.0	−2.1
100 (M)	100 ± 5.0	4.9	5.5	0.2
900 (H)	894 ± 42.6	4.8	4.5	−0.7
5000 (di10-QC)	5180 ± 155	3.0	–	3.6

Table 2
Summery of stability of SS-20 under various storage conditions ($n = 3$).

Storage conditions	Concentrations (ng/mL)		RSD (%)	RE (%)
	Added	Found		
One freeze–thaw cycle	0.80	0.78 ± 0.06	8.4	−3.1
	900	878 ± 22.2	2.5	−2.4
Three freeze–thaw cycles	0.80	0.80 ± 0.10	12.7	0.2
	900	882 ± 35.6	4.0	−2.0
Freezing for 11 days (−70 °C)	0.80	0.79 ± 0.01	1.8	−0.9
	900	836 ± 10.2	1.2	−7.2
Autosampler for 24 h (25 °C)	0.80	0.78 ± 0.05	6.7	−2.7
	900	845 ± 35.1	4.2	−6.1
Short-term (1 h at 4 °C)	0.80	0.74 ± 0.07	9.4	−7.1
	900	900 ± 32.2	3.6	−0.1
Short-term (24 h at 4 °C)	900	606 ± 9.1	1.5	−32.1
Short-term (1 h at 25 °C)	900	663 ± 26.1	3.9	−26.13

3.4.3. Precision and accuracy

The method showed good precision and accuracy. Table 1 summarizes the interday and intraday precisions and accuracy values for SS-20 from QCs. In this assay, the intra day and inter day precisions were measured to be below 10.2% and 5.5%, respectively, with REs from −2.1% to 0.2%. The above values were within the acceptable range, and the method was thus judged as suitably accurate and precise.

The dilution validity experiments were carried out in six replicate by a 10-fold dilution with blank human plasma. The precision was below 3.0%, with RE of 3.6%. The results showed that samples whose concentrations exceeded the upper limit of quantification could be re-analyzed within a 10-fold dilution.

3.4.4. Extraction recovery

The recovery rates of SS-20 extracted from plasma were 56.5 ± 3.7%, 60.7 ± 4.1%, and 57.7 ± 3.3% at concentrations of 0.800, 100, and 900 ng/mL, respectively ($n = 6$). The mean recovery for the IS was 57.7 ± 4.3% ($n = 6$).

3.4.5. Stability

The stability tests of the SS-20 were designed to cover the anticipated conditions of handling non-clinical samples. The results of the stability experiment (Table 2) show that the SS-20 that spiked in the rat plasma were stable for 1 h at 4 °C, for 11 days at −70 °C, and during three freeze–thaw cycles. The stability of SS-20 extracts in the reconstituted extract on autosampler was also observed over a 24 h period. All RE values between post-storage and initial QC samples were within ±15%. However, the SS-20 that was spiked into the rat plasma was unstable for 1 h at ambient temperature, and 24 h at 4 °C.

3.5. Application of the method to a pharmacokinetic study in SD rats

The validated LC–MS/MS method was successfully applied to the pharmacokinetic study of SS-20 in SD rats. To quantify the higher SS-20 concentrations observed in rat plasma samples, the samples were diluted fivefold before analysis. Using this analytical method, we were able to measure the SS-20 concentration for up to 24 h after the intravenous or subcutaneous administration of 1.0 mg/kg SS-20. Fig. 4 shows the profile of the mean SS-20 plasma concentration versus time.

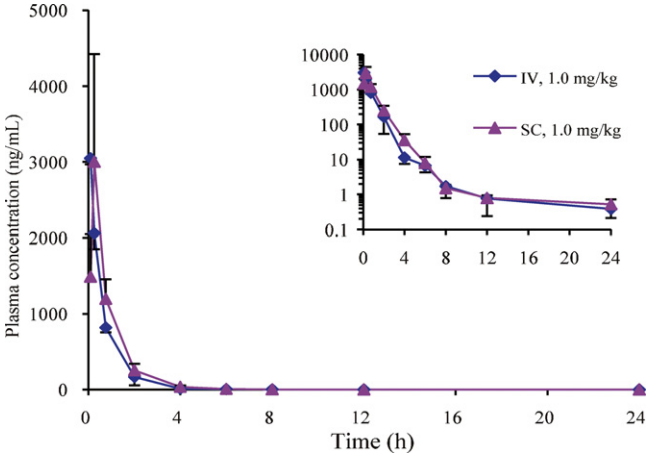

Fig. 4. Mean plasma concentrations of SS-20 after a single injection of 1.0 mg/kg SS-20 given as intravenous injection or subcutaneous injection to SD rats (mean ± SD, $n = 3$).

4. Conclusion

An HILIC–MS/MS method was developed and validated for SS-20 quantification in rat plasma. The use of the HILIC technique solved the poor retention of SS-20 on the chromatographic column and increased the sensitivity of the method by improving the ionization efficiency at high organic solvent concentrations and adopting the SPE in plasma sample preparation. The LLOQ of this method was 0.30 ng/mL, which is sensitive enough for the pharmacokinetic study of low SS-20 doses with good intraday and interday reproducibility for the QCs.

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