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Short communication

Quantitative determination of salidroside in rat plasma by on-line solid-phase extraction integrated with high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

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Abstract

A method for the quantification of salidroside, a major biologically active compound in Rhodiola, in rat plasma by on-line SPE LC/MS/MS in negative electrospray mode was developed and validated. A column-switching instrument and two HPLC pumping systems were employed, and salicin was used as the internal standard. A Waters Oasis HLB extraction column and an Agilent TC-C_{18} analytical column in a column-switching set-up with gradient elution were utilized. The MS/MS ion transitions monitored were *m*/*z* 299.0/119.0 and 285.1/122.9 for salidroside and salicin, respectively. The standard curves were linear within a range of 50–5000 ng/mL using weighted linear regression analysis (1/*x*). The intra- and inter-day coefficients of variance ranged from 1% to 9%. The recovery was above 90%. The freeze/thaw and long-term stability were validated. This method was subsequently applied to a pharmacokinetic study of salidroside in rats.

Keywords: Salidroside; On-line SPE; LC–MS/MS; Pharmacokinetics

1. Introduction

Salidroside (*p*-hydroxyphenylethyl-*O*-*β*-D-glucopyranoside) is one of the major phenolic glycosides of Rhodiola and its content is often used as one of the criteria to evaluate the quality of Rhodiola. It had been found to possess protective effect on anoxia/reoxygenation damages upon myocardium [1], exhibit anti-neuronal apoptosis effect [2], and display antioxidant activity on lipid peroxidation [3].

Many analytical methods for the determination of salidroside in Rhodiola have been reported, such as high-performance liquid chromatography with ultraviolet detection (HPLC–UV) [4–6], thin-layer chromatography (TLC) [7], capillary electrophoresis (CE) [8], high-performance liquid chromatography–mass spectrometry (LC–MS) [9], and high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) [10]. Unfortunately, none of the described methods was suitable for the quantification of salidroside in biological fluids. As the requirement for performing pharmacokinetic studies of traditional Chinese medicines (TCMs) is increasing, the development of suitable method to analyze active compounds in biological fluids is necessary. The use of LC–MS/MS becomes more attractive because of its specificity and sensitivity. The LC–MS/MS in combination with on-line ("column-switching") solid-phase extraction (SPE) could provide straightforward sample work-up and fast capability required for the quantification of active compounds in biological matrices. In the present study, we developed and validated an on-line SPE–LC–MS/MS method for the quantification of salidroside and applied the method to study the pharmacokinetics of salidroside in rats. To our knowledge, this is the first pharmacokinetic study of salidroside in rats that has been fully investigated.

2. Experimental

2.1. Chemicals and reagents

Salidroside was purchased from Fleton Reference Substance (Chengdu, China). The internal standard (IS), salicin, was obtained from Sigma (St. Louis, MO, USA). The purities of both

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standards were all > 98%. Ammonium acetate, formic acid, and other HPLC grades (acetonitrile and methanol) were purchased from Sigma (St. Louis, MO, USA). The deionized water was purified by the Milli-Q water system (Millipore, Bedford, MS, USA).

2.2. Preparation of calibration standards and quality controls

Salidroside and salicin (IS) were weighted and dissolved in 50% methanol to yield 1 mg/mL primary stock solutions. The working stock solutions of salidroside and salicin were prepared by diluting with acetonitrile resulting in concentrations of 10 μg/mL and 1500 ng/mL, respectively. Calibration standards of salidroside were prepared by a serial dilution with blank rat plasma yielding final concentrations of 50, 100, 500, 1000, 1500, 2500, 3500, and 5000 ng/mL. The quality control (QC) samples were prepared by spiking blank rat plasma with independently prepared salidroside standard solutions to give the final concentrations of 200, 2000, and 4000 ng/mL.

2.3. Sample preparation

Aliquots of 100 μL of rat plasma samples (blank plasma, calibration standards, QC, and pharmacokinetic plasma samples) were mixed with 100 μL acetonitrile containing 1500 ng/mL of salicin. The mixture was vortexed for 30 s and then centrifuged at 21,000 × *g* for 20 min in an Eppendorff model 5417c centrifuge at room temperature. The supernatant was transferred to a clean tube and 100 μL supernatant was injected onto on-line SPE–LC–MS/MS. Plasma samples that had concentrations of salidroside above the upper limit of quantification (5000 ng/mL) were diluted proportionally with blank rat plasma prior to extraction with acetonitrile.

2.4. On-line solid-phase extraction and chromatography

A volume of 100 μL of supernatant was injected for sequent on-line SPE–LC–MS/MS analysis. First, the valve stayed in position 1 (Fig. 1(A)), flushing the washing solvent (water, 4.0 mL/min) pumped by the Agilent 1100 series LC System (Palo Alto, CA, USA) through the sample loop onto the Oasis HLB extraction column (25 μm, 1.0 mm × 50 mm; Waters, Milford, USA) for 1.0 min. Simultaneously, the analytical column (Agilent TC-C_{18} reverse-phase column, 5 μm, 4.6 mm × 150 mm) was equilibrated with 10 mM ammonium acetate containing 0.1% formic acid (solvent A):acetonitrile (solvent B) = 70:30 (v/v) at a flow rate of 1.5 mL/min. After 1.0 min, the valve switched to position 2 (Fig. 1(B)), whereupon salidroside was flushed from the extraction column onto the analytical column for final separation and quantification. The following gradient was applied for the analytical column: 0.0–1.5 min, 70% solvent A–30% solvent B; 2.0 min, 30% A–70% B; 2.0–4.0 min, 30% A–70% B; 4.1 min, 70% A–30% B; 4.1–5.0 min, 70% A–30% B. After 3.1 min the valve was switched back to position A. The extraction column was washed with acetonitrile at a flow rate of 4.0 mL/min for 1.0 min, and was subsequently re-equilibrated with water. The total analytical run time was 5.0 min.

2.5. Mass spectrometric conditions

Applied Biosystems Sciex API 3000 tandem mass spectrometer equipped with an ESI in the negative ion mode (Applied Biosystems, Foster City, CA, USA) was applied. The auxiliary gas flow rate was 8.0 L/min. The nebulizing gas, curtain gas and collision gas flow were at the instrument settings of 12, 6 and 12, respectively. The electrospray needle was maintained at −4.5 kV and the heated-capillary temperature was set to 400 °C. Data acquisition was done via multiple reaction monitoring (MRM). Ions representing the $[M-H]^-$ species for both the analyte and IS were selected in MS1 and were collisionally dissociated with nitrogen gas to form specific product ions which were subsequently monitored by MS2. The collision energy was −20 V for the analyte and −18 V for IS. The ions monitored for salidroside (analyte) and salicin (IS) were *m/z* 299.0/119.0 and *m/z* 285.1/122.9, respectively.

2.6. Method validation

Calibration curves were constructed from the working standard solutions of salidroside at a concentration range of

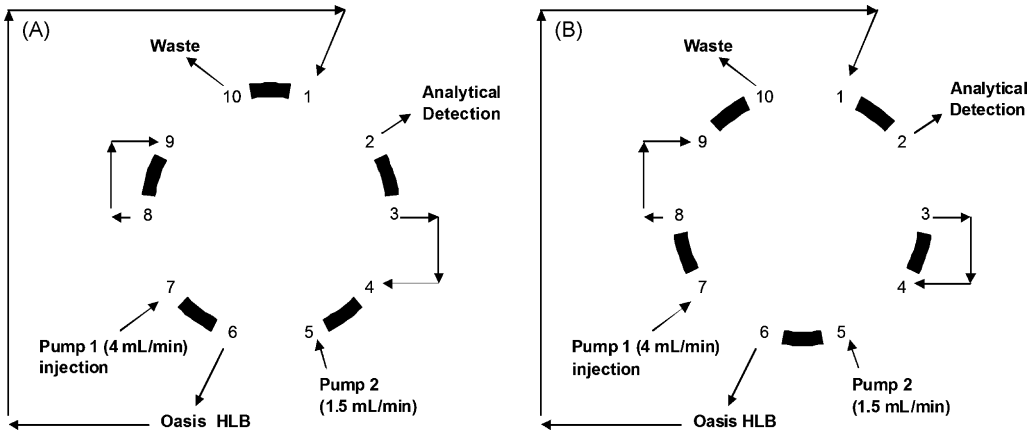

Fig. 1. Column switching scheme applied for on-line solid-phase extraction. (A) Sample is loaded onto the SPE column. (B) Sample is desorbed from the SPE column and flowed onto the analytical column.

50–5000 ng/mL by plotting the peak area ratio (y) of salidroside to the internal standard, salicin, versus salidroside concentration (x). The regression parameters of slope, intercept and correlation coefficient were calculated by weighted ($1/x$) linear regression using Analyst 1.3 software used in AB API3000. The accuracy and precision (presented as relative standard deviation, RSD) of the analytical method were evaluated using QC samples injected onto on-line SPE–LC–MS/MS five times a day for intra-day precision evaluation. The same procedure was performed for five consecutive days to determine inter-day precision. The recoveries of salidroside were determined by comparing the peak areas obtained from the five extracted plasma samples spiked with known amounts of standards with those obtained from the pure compounds of the same concentrations in the solvent. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of the freshly prepared QC samples. The stored QC samples were subjected to three freeze–thaw cycles or were stored at room temperature for 8 h.

2.7. Pharmacokinetic study

The validated method was applied to a pharmacokinetic study of salidroside in rats. A true solution of salidroside was prepared by dissolving an appropriate amount compound in water. Male Sprague–Dawley rats, weighing 250–350 g each (8–10 weeks old), were obtained from BioLASCO, Ilan, Taiwan. The use of the animals followed the guideline approved by the institutional animal care and use committee of the National Health Research Institutes. The compound was administered to groups of five and four rats intravenously (i.v.) and orally (p.o.) at a dose of 5 mg/kg and 25 mg/kg, respectively. The volume of dosing solution administered was adjusted according to the body weight recorded before dose administration. At 0 (prior to dosing), 2, 5, 15, and 30 min and at 1, 2, 4, 6, 8, 12, and 24 h after dosing, blood samples (~200 μL) were collected from each animal via the jugular-vein cannula. Plasma was separated from the blood by centrifugation (14,000 × g for 15 min at 4 °C in a Beckman Model Allegra™ 6R centrifuge) and was stored in a freezer (−60 °C) until analysis. All samples were analyzed for the parent compound by on-line SPE–LC–MS/MS. Plasma concentration data were analyzed using the standard non-compartmental method with the WinNonLin software program (version 3.1, Pharsight, CA, USA).

3. Results and discussion

3.1. Development of the on-line SPE procedures

ESI sources has been proven as a very powerful interface between liquid chromatography and triple quadruple mass spectrometry for the quantitative determination. However, interferences from matrix components (e.g., salts and proteins) appear to have obvious effects on the suppression of ionization of analytes [11]. In order to obtain samples which are clean enough to enter the mass spectrometer, various sample preparation methods have already been applied [12–16]. As compared to liquid–liquid extraction, protein precipitation, and off-line SPE, on-line SPE offers advantage in term of being easier to handle and more time-efficient [17–23]. To extend the life of SPE extraction column, a simple protein precipitation with acetonitrile was applied prior to injection. No blockage was observed in the extraction column. This indicated that the on-line SPE procedure was acceptable.

3.2. Mass spectrometry

$[M+Na]^+$ and $[M-H]^-$ ions of salidroside were observed by FIA experiments using positive and negative ion modes, respectively. Although the sodiated molecular ions $[M+Na]^+$ appeared more abundant ions in the positive ion mode than the deprotonated molecular ions $[M-H]^-$ in the negative ion mode at the same concentration level, the selection of the $[M+Na]^+$ ion as the quantitative ion was not suitable as the sodium content in the analytical system might vary to some extents [24]. Thus, the analysis of salidroside was performed in the negative ion mode. The MS/MS parameters were optimized to give the maximum response for the salidroside and salicin (IS). The mass spectra of salidroside $[M-H]^-$ m/z 299.0 and salicin $[M-H]^-$ m/z 285.1 are shown in Fig. 2. The MS/MS transition 299.0/119.0 and 285.1/122.9 for salidroside and salicin were selected since the ion scan product presented at the highest abundance.

3.3. Chromatography

In order to develop an optimum method with a high sample throughput, symmetrical peak shapes and short retention times were attained. The chromatographic retention times of salidroside and salicin were 2.9 and 2.8 min, respectively (Fig. 3). The total run time was 5 min. LC–MS/MS is considered as a powerful and selective instrument, which allows the quantification of even some co-eluenting peaks. Thus, no further attempted was pursuit to achieve a complete chromatographic separation of compounds [25].

3.4. Method validation

These calibration curves were found to be linear at the concentration range 50–5000 ng/mL in rat plasma with a correlation coefficient ($r>0.99$). The typical calibration curve is $Y=(0.00424\pm0.0002)X+(-0.0406\pm0.0016)$ indicating good linear relationships between the peak areas and concentrations ($r=0.9982$). The limit of quantification (LOQ) was 50 ng/mL in 100 μL of rat plasma. The accuracy and precision at LOQ were 107 ± 6% and 4% ($n=5$), respectively. The intra-day accuracy ranged from 93% to 108% (Table 1). The intra-day precision of salidroside for the three concentrations examined was 1%, 5%, and 5%, respectively. The inter-day accuracy ranged from 94% to 107%, and the inter-day precision ranged form 2% to 9%. Due to lack of a suitable stable labeled form of salidroside, a structurally related derivative, salicin was used as the internal standard. The overall recovery of salidroside from rat plasma was 90%, 102% and 114% at concentrations of 200, 2000, and 4000 ng/mL, respectively. The recoveries of the three

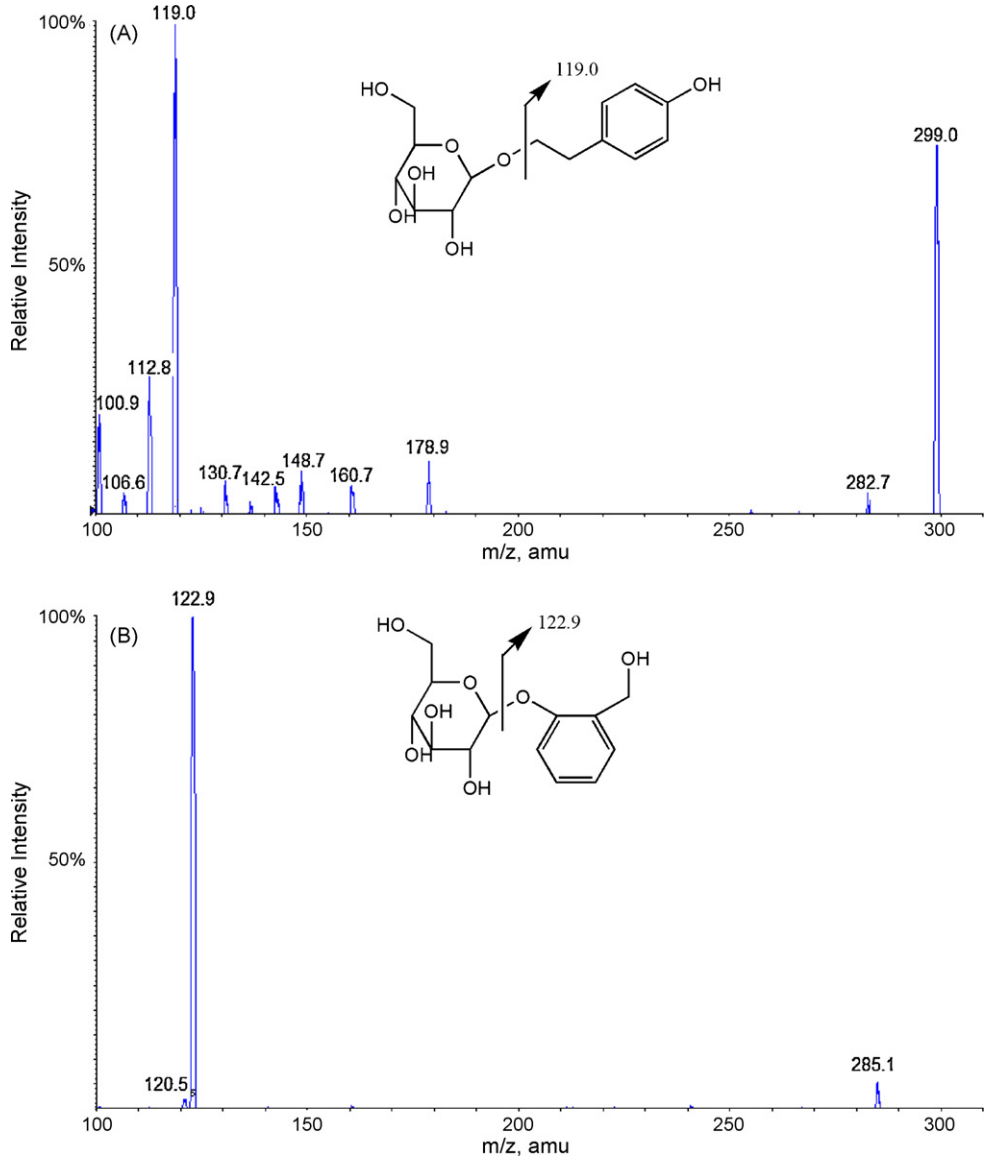

Fig. 2. Product ion mass spectra of $[M-H]^-$. (A) Salidroside ($[M-H]^-$, *m/z* 299.0). (B) Salicin ($[M-H]^-$, *m/z* 285.1).

examined concentrations were high (ranged from 90% to 114%). These results indicated no significant effect of endogenous substances on the ionization of salidroside due to the rigorous washing procedure suggesting that matrix effects were likely to be fairly small. The stability of salidroside in rat plasma under different storage conditions is summarized in Table 2. There was no significant degradation observed at ambient temperature for 8 h and during the freeze–thaw cycles for salidroside in rat plasma samples in which the concentrations deviated by not more than 5% relative to the reference nominal concentrations.

3.5. Pharmacokinetic study

The method was applied to the pharmacokinetic study of salidroside in rats. The mean plasma concentration–time profiles are shown in Fig. 4. The pharmacokinetic parameters of salidroside after intravenous and oral administration to rats are

Table 1
Intra- and inter-day accuracy and precision for salidroside in rat plasma

Nominal concentration (ng/mL)	Intra-day precision ($n=5$)			Inter-day precision ($n=5$)		
	200	2000	4000	200	2000	4000
Measured concentration (ng/mL)	186 ± 2	2053 ± 99	4300 ± 226	188 ± 4	2127 ± 200	4277 ± 390
Accuracy (%)	93 ± 1	103 ± 5	108 ± 6	94 ± 2	106 ± 10	107 ± 10
RSD (%)	1	5	5	2	9	9

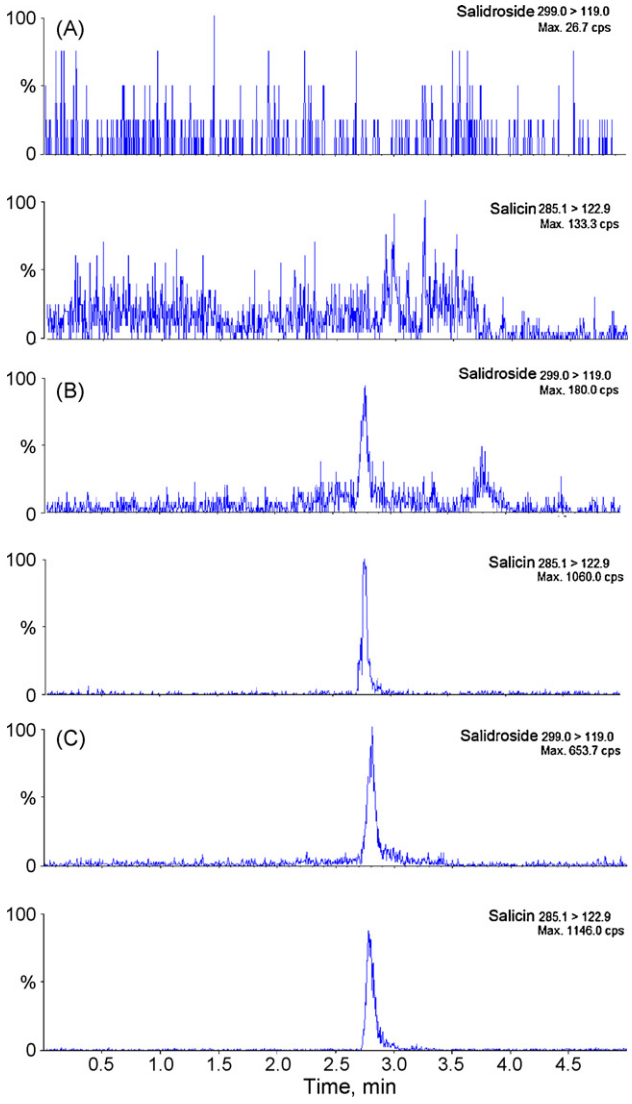

Fig. 3. Representative MRM chromatograms. (A) Blank rat plasma. (B) Blank rat plasma spiked with salidroside (50 ng/mL) and internal standard (1500 ng/mL). (C) Rat plasma sample 1 h after intravenous administration to a rat with 5 mg/kg dose of salidroside. The plasma concentration of salidroside was 112 ng/mL.

summarized in Table 3. The plasma concentrations of salidroside declined rapidly following intravenous dosing. Salidroside showed high systemic clearance (Cl: 53 ± 17 mL/min/kg) and moderate volume of distribution at the steady state (V_{ss}: 0.8 ± 0.4 L/kg). The apparent elimination half-life ($t_{1/2}$) and the mean resident time (MRT) were 0.5 ± 0.2 and 0.2 ± 0.1 h, respectively. The corresponding value for $AUC_{(0-\infty)}$ was 1780 ± 660 ng/mL × h. After oral dosing, salidroside showed rapid oral absorption in rats, with a short T_{max} of 30 min. The C_{max} and AUC of salidroside were approximately 6493 ± 1768 ng/mL and 8486 ± 2441 ng × h/mL, respectively. The mean residence time (MRT) and the terminal half-life ($t_{1/2}$) were 1.1 ± 0.2 h and 1.1 ± 0.7 h, respectively. The mean absorption time (MAT) was 0.9 h. The oral bioavailability of salidroside was high (~98%).

Table 2
Stability of salidroside in rat plasma

Storage condition	Nominal concentration (ng/mL)		
	200	2000	4000
Measured concentration (ng/mL)			
Three freeze–thaw cycle stability (−37 °C)			
Mean	188 ± 4	2277 ± 60	4547 ± 249
RSD (%)	2	3	5
Post-preparative stability (8 h at room temperature)			
Mean	180 ± 1	2123 ± 104	4023 ± 57
RSD (%)	0	5	2

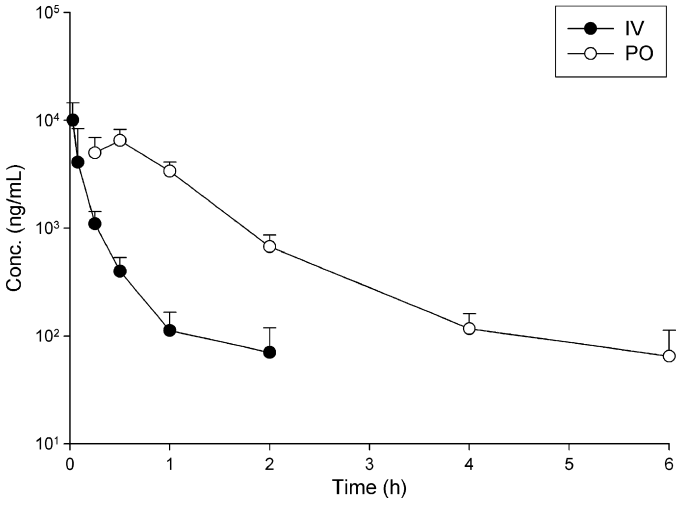

Fig. 4. Mean plasma concentration–time profiles of salidroside after intravenous and oral doses to rats.

Table 3
Pharmacokinetic parameters of salidroside after a single intravenous and oral administration in rats

Parameter	Unit	IV	PO
n		5	4
Dose	mg/kg	5	25
$t_{1/2}$	h	0.5 ± 0.2	1.1 ± 0.7
MRT	h	0.2 ± 0.1	1.1 ± 0.2
MAT	h	–	0.9[a]
Clearance	mL/min/kg	53 ± 17	–
V_{ss}	L/kg	0.8 ± 0.4	–
C_{max}	ng/mL	–	6493 ± 1768
T_{max}	h	–	0.5 ± 0.0
$AUC_{(0-\infty)}$	ng/mL × h	1780 ± 660	8486 ± 2441
Oral bioavailability (F)	%		98.0[b]

[a] $MAT = MRT_{PO} - MRT_{IV}$.
[b] $F\ (\%) = (AUC_{PO(0-\infty)}/\text{PO dose}) \times 100/(AUC_{IV(0-\infty)}/\text{IV dose})$.

4. Conclusion

A rapid and throughput on-line SPE–LC–MS/MS method was developed for the determination of salidroside in rat plasma. This method has high accuracy, precision, and recovery. Moreover, this method was successfully employed for the pharmacokinetic studies of salidroside in rats following intravenous and oral administration. This is the first method to determine salidroside in rat plasma by on-line SPE–LC–MS/MS.

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