Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 861 (2008) 10–15
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of a liquid chromatographic/electrospray ionization mass spectrometric method for the determination of salidroside in rat plasma: Application to the pharmacokinetics study

Sen Yu, Li Liu, Tao Wen, Yuchun Liu, Dianlei Wang, Yuxian He, Yan Liang, Xiaodong Liu *, Lin Xie, Guangji Wang, Wenzhi Wei

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, PR China

Received 28 June 2007; accepted 23 November 2007
Available online 4 December 2007

Abstract

A sensitive and specific liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method has been developed and validated for the identification and quantification of salidroside, a major active constituent from *Rhodiola rosea* L., in rat plasma using helicid as an internal standard. The method involves a simple single-step liquid–liquid extraction with *n*-butanol. The analytes were separated by isocratic gradient elution on a Shim-pack ODS (4.6 μm, 250 mm × 2.0 mm i.d.) column and analyzed in selected ion monitoring (SIM) mode with a negative electrospray ionization (ESI) interface using the respective $[M+Cl]^-$ ions, *m*/*z* 335 for salidroside, *m*/*z* 319 for internal standard. The method was validated over the concentration range of 5–2000 ng/mL for salidroside. Within- and between-batch precision (R.S.D.%) were all within 6% and accuracy ranged from 96 to 112%. The lower limits of quantification was 5 ng/mL. The extraction recovery was on average 86.6% for salidroside. The validated method was used to study the pharmacokinetic profile of salidroside in rat plasma after intravenous and oral administration of salidroside. The bioavailability of salidroside in rats is 32.1%.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Liquid chromatography/mass spectrometry; Salidroside; Rat plasma; Pharmacokinetics; Bioavailability

1. Introduction

Rhodiola rosea L. (family Crassulaceae), the most important species of the genus *Rhodiola*, is a shrub that is widely distributed at high altitudes in the Artic and in mountainous regions throughout Europe and Asia, where it is also known as "golden root" or "artic root" [1]. This plant is very popular in traditional medicine, with a reputation for alleviating emotional, mental and physical disorders [1–5].

R. rosea L. is used in traditional folk medicine to stimulate the nervous system, to decrease depression, to enhance work performance, longevity and resistance to high altitude sickness, and to treat fatigue and symptoms of asthenia subsequent to intense physical and psychological stress [6–8]. Therefore, due to its ability to increase the resistance of an organism to environmental stress factors and to avoid damage from such factors, *R. rosea* L. has been defined as an "adaptogen" [5,9–11].

Salidroside (*p*-hydroxyphenethyl-β-D-glucoside, structure shown in Fig. 1A), which is a major active constituent from *R. rosea* L., has been reported to have various pharmacological properties including anti-aging, anticancer, anti-inflammation, hepatoprotective and antioxidative effects [12–15]. For example, salidroside has been found to be protective against neuron cell death induced by glutamate and hypoxia/hypoglycemia [16–17], and against mitochondria dysfunction induced by sodium azide [18].

In 2006, Lin et al. described a method using radioiodine-labeled salidroside to investigate its distribution in mice [19]. In this year, Chang et al. reported a quantitive determination method of salidroside in rat plasma by on-line-SPE integrated

* Corresponding author. Tel.: +86 25 8327 1006; fax: +86 25 8530 6750.
E-mail address: xdliu@cpu.edu.cn (X.D. Liu).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.11.035

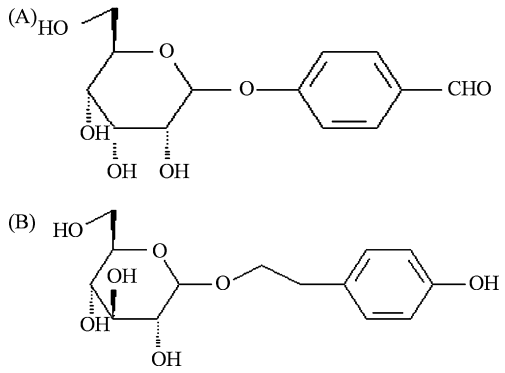

Fig. 1. Chemical structures of internal standard helicid (A) and salidroside (B).

with LC/MS/MS [20]. Radiation is a potential health risk for humans, while on-line-SPE integrated with MS/MS instruments could be too expensive to justify for use in routine measurements in the pre-clinical laboratory. Moreover, the lower limit of quantification (LLOQ; 50 ng/mL) of the latter was also not sensitive enough to fully evaluate the pharmacokinetics of salidroside in pre-clinical or clinical studies. Thus, it was necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy, precision and safety. The potentially large numbers of samples in pre-clinical studies need a rapid and reliable assay. An ideal method should have simple sample preparation, fast on-column separation, and sensitive and specific detection. LC/MS has become an analytical tool that meets most of the above needs.

In the present work, isolation of salidroside was achieved by a single-step liquid–liquid extraction (LLE) with *n*-butanol, followed by solvent evaporation, re-dissolution of the residue, and injection onto the chromatographic column. An electrospray ionization (ESI) interface was used because it provided more sensitivity and better reproducibility for salidroside. The analytical procedure was fully validated and successfully used to assess the pharmacokinetics of salidroside in rats.

2. Experimental

2.1. Material and methods

2.1.1. Standards and reagents

The reference standard of salidroside was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Salidroside injection was from HengJi Medicine Company (Sichuan Province, PR China). The internal standard (IS), helicid (structure shown in Fig. 1B), was kindly provided by Prof. Xiaoquan Liu (Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, PR China). Methanol was of chromatographic grade (Merck Company Inc., Germany). All other reagents were of analytical grade. Milli-Q water (Millipore, Bedford, MA) was used throughout the study.

2.1.2. Liquid chromatographic and mass spectrometric conditions

The LC/MS system consisted of a Shimadzu LC-10AD HPLC series liquid chromatograph and a Shimadzu LC/MS-2010A single quadrupole mass spectrometer equipped with an electrospray ionization interface and a Q-array-Octapole-Quadrupole mass analyzer. Shimadzu LCMS solution Version 2.04 was used for data acquisition and processing.

LC separation was achieved using a Shim-pack ODS (4.6 μm, 250 mm × 2.0 mm i.d., Shimadzu) column maintained at 40 °C. A mobile phase composed of methanol–100 μM ammonium chloride (25:75, v/v) at a flow rate of 0.2 mL/min was used to obtain the baseline separation of both analytes.

The effluent from the HPLC column was directed into the ESI probe. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The curve dissolution line (CDL) temperature and the block temperature were maintained at 250 and 200 °C, respectively. The probe voltage (capillary voltage), CDL voltage and detector voltage were fixed at −4.5 kV, 10 V and 1.6 kV, respectively. Vacuum was created by a Turbo molecular pump (Edwards 28, UK). Liquid nitrogen (99.995%, Nanjing University, PR China) was used as the source of nebulizer gas (1.5 L/min) and drying gas (curtain gas) (4.0 L/min). Analytes were quantitated in selected ion monitoring (SIM) mode. $[M+Cl]^- = 335$ for salidroside and 319 for helicid (IS) were selected as detecting ions. Mass spectra were obtained at a dwell time of 0.2 s in SIM and 1 s in scan mode.

2.1.3. Standard and quality control sample preparation

Stock solutions containing 1 mg/mL of each reference compound was prepared in methanol and stored at 4 °C until use. Working solutions, ranging from 50 to 20,000 ng/mL, were prepared by serial dilution with methanol. A solution containing 1 μg/mL IS was also prepared in methanol.

The samples for standard calibration curves were prepared by spiking the blank rat plasma (100 μL) with 10 μL of the appropriate working solutions to yield the following concentrations: 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL. Quality control (QC) samples were prepared from blank plasma at concentrations of 10, 100 and 1000 ng/mL.

2.1.4. Sample preparation

QC samples, calibration standards, and rat plasma samples were extracted by the liquid–liquid extraction (LLE) technique with *n*-butanol.

A volume of 100 μL of drug-free rat plasma was added to a disposable Eppendorf tube, followed by spiking with 10 μL of the standard working solution and 10 μL of IS working solution, respectively. The mixture was vortexed for 30 s using a vortex mixer (Scientific Industries Inc., USA). Then, a single-step LLE was adopted to extract both the analytes from the rat plasma. For this, 1 mL of *n*-butanol were added to each tube followed by vortexing for 5 min. The well-vortexed solutions were then centrifuged at 15,000 rpm for 10 min and 800 μL of the upper organic layer was transferred to a new Eppendorf tube and evaporated to dryness in a Thermo Savant SPD 2010 SpeedVac system (Thermo Electron Corporation, USA) set at 45 °C. The residues were then reconstituted in 200 μL mobile phase followed by centrifugation at 22,000 rpm for 10 min before analysis. An aliquot of 5 μL was injected into the LC/MS system.

When the concentration of salidroside in rat plasma is over the linear calibration curve range, appropriate dilution of the plasma sample with blank rat plasma is needed. The blank plasma for the dilution was the same as those used to prepare calibration standards.

2.2. Method validation

The method validation assays were carried out according to the currently accepted U.S. Food and Drug Administration (FDA) bioanalytical method validation guidance [21].

2.2.1. Assay specificity

The specificity of the method was evaluated by analyzing blank plasma samples from six rats. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectroscopic conditions and compared with those obtained with an aqueous solution of the analyte at a concentration near the lower limit of quantification (LLOQ).

The matrix effect on the ionization of the analytes was evaluated by comparing the peak areas of the analytes resolved in the blank sample (the final solution of blank plasma after extraction and reconstitution) with that resolved in the mobile phase. Three different concentration levels of salidroside (10, 100 and 1000 ng/mL) and 100 ng/mL of the IS were evaluated by analyzing five samples at each level. The blank plasmas used in this study were six different batches of blank rat plasmas. If the ratio is <85% or >115%, an exogenous matrix effect is implied.

2.2.2. Linearity

Calibration curves of nine concentrations of salidroside from 5 to 2000 ng/mL were extracted and assayed. Blank plasma samples were analyzed to confirm the absence of interferences but were not used to construct the calibration function.

2.2.3. Precision and accuracy

The precision of the assay was determined from the QC plasma samples by replicate analyses of four concentration levels of salidroside (5, 10, 100 and 1000 ng/mL). Within-batch precision and accuracy were determined by repeated analyses of the group of standards on one batch ($n = 5$). Between-batch precision and accuracy were determined by repeated analyses on three consecutive days ($n = 5$ series per day). The concentration of each sample was determined using the calibration curve prepared and analyzed on the same batch.

2.2.4. Extraction recovery

The extraction recoveries of salidroside were determined at low, medium and high concentrations. Recoveries were calculated by comparing the analyte/IS peak area ratios (R_1) obtained from extracted plasma samples with those (R_2) from the standard solutions at the same concentration.

2.2.5. Stability

Freeze and thaw stability. QC plasma samples at three concentration levels were stored at the storage temperature (−20 °C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated twice, and the samples were analyzed after three freeze (−20 °C)–thaw (room temperature) cycles.

Short-term temperature stability. QC plasma samples at three concentration levels were kept at room temperature for a period that exceeded the routine preparation time of the samples (around 6 h).

Long-term stability. QC plasma samples at three concentration levels kept at low temperature (−20 °C) were studied for a period of 2 weeks.

Post-preparative stability. The autosampler stability was conducted by reanalyzing extracted QC samples kept under autosampler conditions (4 °C) for 12 h.

Stock solution stability. The stability of salidroside and the IS working solutions were evaluated at room temperature for 4 days.

2.2.6. Standard curves and quality control samples in each batch

Standard curves in each analytical run were used to calculate the concentrations of salidroside in the unknown samples in the run. Quality control samples were prepared along with the unknown samples in the same batch and analyzed in the middle of the run.

The QC samples of salidroside in five duplicates at four concentrations (5, 10, 100 and 2000 ng/mL).

2.3. Safety considerations

The method required no specific safety precautions. Universal precautions for the handling of chemicals and biofluids were applied.

2.4. Application to pharmacokinetic study

The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Twelve male Sprague–Dawley rats (200–220 g) were obtained from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, PR China). The rats were maintained in an air-conditioned animal quarter at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%, free access to water, and feeding with a laboratory rodent chow (Nanjing, PR China).

The animals were acclimatized to the facilities for 5 days, and then fasted, free access to water for 12 h prior to experiment. Salidroside was administered to the rats (12 mg/kg body weight) by intravenous injection or oral gavage. Blood samples (100 μL at the timepoints needed to be diluted, 300 μL at the other timepoints) were obtained from the oculi chorioideae vein before dosing and subsequently at 5, 10, 20, 30, 60, 90, 120 and 180 min following intravenous administration and at 5, 10, 15, 30, 60, 90, 120, 180 and 240 min after oral administration, transferred to a heparinized Eppendorf tube and centrifuged at 4000 rpm for 10 min. The plasma obtained was frozen at −20 °C until analysis.

To determine the pharmacokinetic parameters of salidroside, the concentration–time data were analyzed by non-compartmental methods using the DAS Software (Version 1.0, Medical College of Wannan, PR China). C_{max} values were obtained directly from the observed concentration versus time data. All results were expressed as arithmetic mean ± standard deviation (S.D.). Bioavailability is calculated according to the following equation:

$$\text{bioavailability}\,(F) = \frac{\text{AUC}_{0-\infty}(\text{p.o.})}{\text{AUC}_{0-\infty}(\text{i.v.})}$$

3. Results and discussions

3.1. Method development

During the scan of salidroside, the ESI-MS system revealed the presence of $[M-H]^-$ as well as $[M+Cl]^-$ ion. The chromatographic peak areas were 2–4 times higher for $[M+Cl]^-$ than for $[M-H]^-$ ion. Therefore, $[M+Cl]^-$ ion was employed for method validation.

Although higher acetonitrile ratios had been tried for decreasing the analysis time, the analytes could not be totally separated from the endogenous plasma components. The optimization of chromatography can also reduce ion suppression and increase signal. Addition of 100 μM ammonium chloride to the mobile phase was found to be an important factor for acquiring the high sensitivity. When the ion adduct $[M+Cl]^-$ was selected for determination, the addition of ammonium chloride caused a significant sensitivity increase. One hundred micromolars of ammonium chloride was the concentration after optimization using a series of concentration.

Helicid was chosen for quantification as the IS due to its similarity with the analytes in structure, chromatographic behavior, mass spectrographic behavior and stability [22].

LLE was advantageous because this technique not only extracted the analyte and IS with sufficient efficiency and specificity, but also minimized the experimental cost. Ethyl acetate, diethyl ether and *n*-butanol were all tested as extraction solvent, and *n*-butanol was finally adopted because of its high extraction efficiency.

3.2. Method validation

Both samples were found to be free from interferences with the compounds of interest. All the ratios of the peak area resolved in the blank sample, compared with that resolved in the mobile phase, are between 85 and 115%, which means no matrix effect is observed for salidroside and helicid in this method. Fig. 2 shows negative ion electrospray mass scan spectra of the analyte and IS. Representative SIM chromatograms are shown in Fig. 3, indicating no endogenous peaks at the retention positions of salidroside or the IS (helicid).

The calibration curves were prepared daily which showed good linearity in the range 5–2000 ng/mL for salidroside. The mean regression equation from five replicate calibration curves on different days was: $R = 0.0059(\pm 0.00002)C + 0.0241$ (± 0.00291), $r = 0.9999$, where R corresponds to the peak area ratio of salidroside to the IS, and C (ng/mL) refers to the concentration of salidroside added to plasma. Results of the calibration curves for salidroside LC–MS determination are given in Table 1.

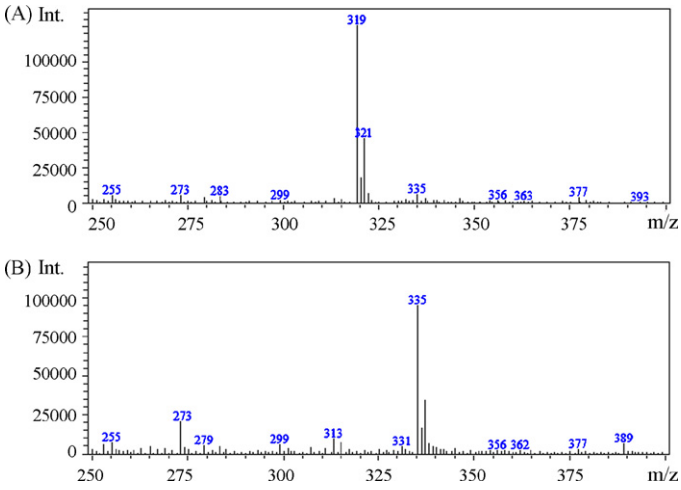

Fig. 2. Negative ion electrospray mass scan spectra of internal standard helicid (A) and salidroside (B).

The lower limit of quantification for salidroside was proved to be 5 ng/mL (LLOQ) and the lower limit of detection (LLOD) was 1 ng/mL.

Data for within-batch and between-batch precision and accuracy of the method for determination of salidroside are presented in Table 2. The accuracy deviation values are within 12% of

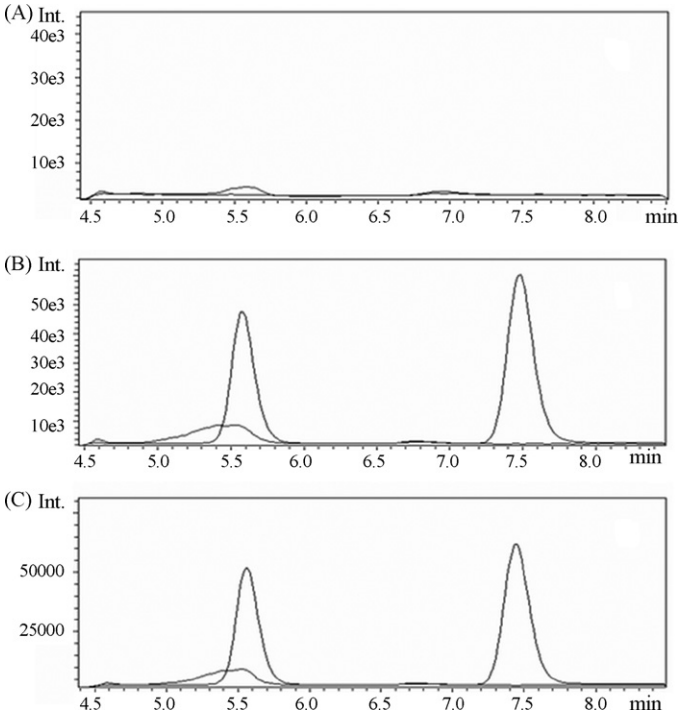

Fig. 3. SIM chromatograms of (A) blank plasma; (B) blank plasma spiked with salidroside (200 ng/mL) and helicid (100 ng/mL); (C) samples 30 min after intravenous administration. The retention times of helicid and salidroside were 5.6 and 7.5 min.

Table 1
Results of calibration curves for salidroside LC–MS determination ($n = 5$)

	Spiking plasma concentration (ng/mL)								
	5.0	10	20	50	100	200	500	1000	2000
Mean	4.8	9.1	20.6	52.3	103.0	206.4	494.2	988.3	2006.5
R.S.D. (%)	5.2	1.5	2.4	6.7	4.0	4.0	3.1	2.9	0.6
Accuracy (%)	95.2	91.4	102.8	104.5	102.9	103.2	98.8	98.8	100.3

Table 2
Accuracy and precision for the analysis of salidroside ($n = 5$)

Spiking plasma concentration (ng/mL)	Within-batch		Between-batch	
	Precision (CV, %)	Accuracy (%)	Precision (CV, %)	Accuracy (%)
5	3.0	99.6	5.7	111.6
10	3.8	102.3	3.7	109.9
100	3.0	105.8	3.2	105.1
2000	4.4	96.5	5.6	102.9

Table 3
Recovery of salidroside from plasma ($n = 5$)

Spiking plasma concentration (ng/mL)	Recovery (mean ± S.D., %)	R.S.D. (%)
10	87.9 ± 1.6	1.9
100	88.2 ± 3.7	4.2
1000	83.7 ± 2.5	2.9

the actual values. The precision determined at each concentration level does not exceed 6% of the relative standard deviation (R.S.D.). The results revealed good precision and accuracy.

The extraction recovery determined for salidroside was shown to be consistent, precise and reproducible. Data are shown in Table 3. The extraction recovery of IS was more than 70%.

Table 4 summarizes the freeze and thaw stability, short-term stability, long-term stability and post-preparative stability data of salidroside. All the results showed good stability during these tests and there were no stability related problems during the routine analysis of samples for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the working solutions was tested at room temperature for 4 days. On the basis of the results obtained, these working solutions were found to be stable for 4 days.

Table 4
Data showing the stability of salidroside in rat plasma at different QC levels ($n = 5$)

	Accuracy (mean ± S.D., %)		
	10 ng/mL	100 ng/mL	1000 ng/mL
Freeze and thaw stability	106.8 ± 5.2	105.6 ± 3.6	94.9 ± 2.1
Short-term stability	104.4 ± 4.7	103.0 ± 5.6	95.5 ± 3.7
Long-term stability	105.3 ± 5.7	102.5 ± 2.5	91.7 ± 3.5
Post-preparative stability	97.3 ± 5.2	99.3 ± 4.9	102.4 ± 3.2

10, 100 and 1000 ng/mL are spiking plasma concentrations.

3.3. Pharmacokinetics study

The method described above was successfully applied to a pharmacokinetics study in which plasma concentrations of salidroside were determined for 3 h after intravenous administration and for 4 h after oral administration both at a dose of 12 mg/kg. The pharmacokinetic profiles of salidroside are shown in Fig. 4. Based on these results the PK parameters were calculated. The calculated pharmacokinetic parameters are summarized in Table 5.

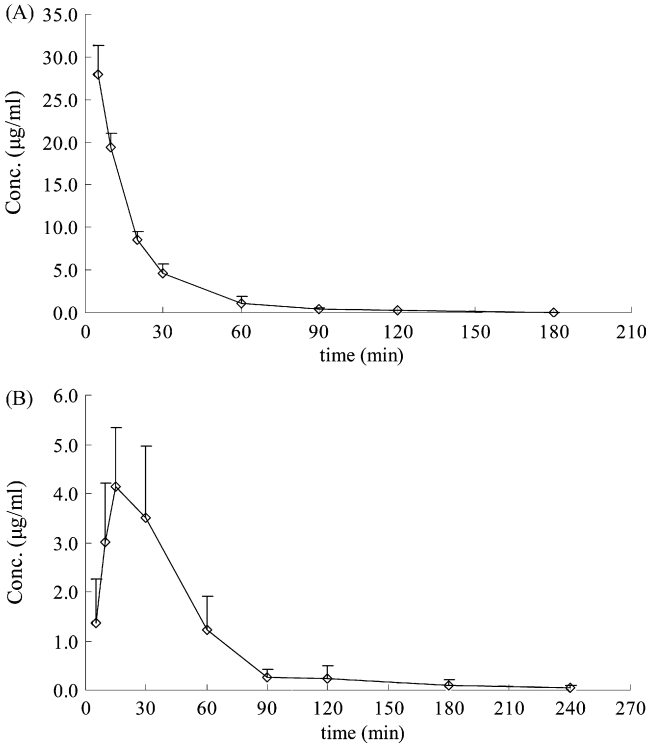

Fig. 4. Mean plasma concentration–time profiles of salidroside in rats after intravenous (A) and oral (B) administration both of 12 mg/kg, each point and bar represents the mean ± S.D. ($n = 6$).

Table 5
Pharmacokinetic parameters of salidroside following intravenous and oral administration both at a dose of 12 mg/kg, each value represents the mean ± S.D. ($n = 6$)

Pharmacokinetic parameters	i.v. administration	Oral administration
C_{max} (μg/mL)	27.9 ± 3.4	4.3 ± 1.1
MRT (min)	17.9 ± 2.5	41.7 ± 17.7
CL (L/(kg min))	0.021 ± 0.003	0.098 ± 0.044
AUC_{0-180} (μg min/mL)	624.0 ± 84.4	202.6 ± 77.2
$AUC_{0-\infty}$ (μg min/mL)	625.4 ± 84.7	205.0 ± 79.0
F (%)	32.1 ± 9.6	

4. Conclusions

This paper described a sensitive, specific, accurate and precise HPLC–ESI-MS method for the determination of salidroside in rat plasma. Good linearity was observed in the range 5–2000 ng/mL and the lower limit of quantification (5 ng/mL) makes this method of real practical use for pharmacokinetic, bioavailability and bioequivalence studies.

The method was successfully applied to a pharmacokinetic study of salidroside in rats. It is the first study, as far as we know, of LC/MS method for determination of salidroside concentration in vivo.

References

[1] C. German, Z. Ramazanov, M. Bernard Suarez, Artic Root (*Rodiola rosea*): The Powerful Ginseng Alternative, Kensington Publishing Corp., New York, 1999.
[2] A.A. Spasov, G.K. Wikman, V.B. Mandrikov, I.A. Mironova, V.V. Neumoin, Phytomedicine 7 (2000) 85.
[3] V.A. Shevtsov, B.I. Zholus, V.B. Shervarly, V.I. Vol'skij, Y.P. Korovin, M.P. Khristich, N.A. Roslyakova, G. Wikman, Phytomedicine 10 (2003) 95.
[4] A.S. Saratikov, E.A. Krasnov, *Rhodiola rosea* (Golden Root): A Valuable Medicinal Plant, Tomsk University Press, Tomsk, 2004.
[5] A. Panossian, H. Wagner, Phytother. Res. 19 (2005) 819.
[6] G.S. Kelly, Altern. Med. Rev. 6 (2001) 293.
[7] R.P. Brown, P.L. Gerbarg, Z. Ramazanov, HerbalGram 56 (2002) 40.
[8] B.W. Zhu, Y.M. Sun, X. Yun, S. Han, M.L. Piao, Y. Murata, M. Tada, Biosci. Biotechnol. Biochem. 67 (2003) 1930.
[9] H. Wagner, H. Norr, H. Winterhoff, Phytomedicine 1 (1994) 63.
[10] A. Panossian, G. Wikman, H. Wagner, Phytomedicine 6 (1999) 287.
[11] A. Panossian, Altn. Complement. Ther. 9 (2003) 327.
[12] A.M. Diaz Lanza, M.J. Abad Martinez, L. Fernandez Matellano, C. Recuero Carretero, L. Villaescusa Castillo, A.M. Silvan Sen, P. Bermejo Benito, Planta Med. 67 (2001) 219.
[13] I.N. Iaremii, N.F. Grigor'eva, Farmakol 65 (2002) 57.
[14] D. Kanupriya, M. Prasad Sai Ram, R. Kumar, R.C. Sawhney, S.K. Sharma, G. Ilavazhagan, D. Kumar, P.K. Banerjee, Mol. Cell. Biochem. 275 (2005) 1.
[15] A. Kucinskaite, V. Briedis, A. Savickas, Medicina (Kaunas) 40 (2004) 614.
[16] L.L. Cao, G.H. Du, M.W. Wang, J. Asian Nat. Prod. Res. 8 (2006) 159.
[17] W.S. Zhang, L.Q. Zhu, F.L. Niu, R.C. Deng, C.X. Ma, Zhongguo Zhong Yao Za Zhi. 29 (2004) 459.
[18] L.L. Cao, G.H. Du, M.W. Wang, Yao Xue Xue Bao 40 (2005) 700.
[19] X.F. Lin, H.X. Yu, C. Tan, L. Zhang, B. Chen, R.J. Zhang, X.J. Xu, G.X. Cao, Nucl. Techniq. 12 (2006) 913.
[20] Y.W. Chang, H.T. Yao, S.H. Hsieh, T.J. Lu, T.K. Yeh, J. Chromatogr. B 857 (2007) 164.
[21] US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Guidance for Industry, Bioanalytical Method Validation, 2001.
[22] Y.W. Jia, G.J. Wang, H.T. Xie, X.C. Dai, Y.H. Wang, W. Wang, M.J. Xu, R. Wang, C. Yao, J. Chromatogr. B 847 (2007) 72.