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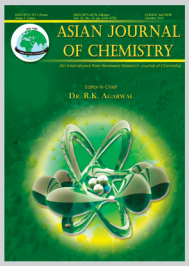

Pharmacokinetics of Ginsenosides Rg1, Re and Notoginsenoside R1 in Rats by an *in vivo* Microdialysis Coupled with Liquid Chromatography/Mass Spectrometry

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The pharmacokinetic profiles of ginsenosides Rg1, Re and notoginsenoside R1, major active components of Xuesaitong injection, were firstly studied in Sprague-Dawley rats by the microdialysis technique, which is semi-invastive and need less animals. Without pretreatment, the dialysate samples were eluted on a Zorbax Eclipse SB-C_{18} (100 mm × 2.1 mm, 3.5 μm) column and quantified by monitoring the ions at m/z 859 (GRg1), m/z 931 (NR1) and m/z 945 (GRe) in negative electrospray ionization mass spectrometry. Pharmacokinetic parameters were calculated by fitting the accumulative area under curve to time, which avoids the system deviation about parameter estimation. Their disposition kinetics could be adequately described by a two- or one-compartment model with first-order elimination. Comparing with the conventional serial blood sampling, microdialysis sampling approach could potentially yield more reliable pharmacokinetic data due to the intensive sampling time points, especially for rapidly eliminated drugs.

Key Words: Liquid chromatography-mass spectrometry, Microdialysis, Xuesaitong injection, Pharmacokinetics.

INTRODUCTION

The root of *Panax notoginseng*, commonly known as Sanqi or Tianqi in China, is an important component in various prescriptions in traditional Chinese medicine. Clinical studies confirmed that it possesses anticarcinogenic[1] and hepatoprotective[2] properties, as well as protective effects on cardiovascular and cerebrovascular diseases[3-5]. Total Panax notoginsenosides (TPNS) including ginsenosides and notoginsenosides have been regarded as the principal components manifesting the pharmacological activities. The pharmacokinetic studies of total Panax notoginsenosides were mainly focused on ginsenoside Rg1 (GRg1), ginsenoside Re (GRe) and notoginsenoside R1 (NR1) for their high content in total Panax notoginsenosides. Since these three saponins were easily destroyed in gastrointestinal tract, metabolized by intestinal microflora and excreted from bile or urine[6,7], the absolute bio-availabilities of GRg1, GRe and NR1 were of 6.06, 7.06 and 9.29%, respectively[8]. The possible application of these compounds in therapy was hampered by their poor absorption. Xuesaitong injection has emerged to solve this problem. It was composed of total saponins extracted from Sanqi and widely used for the treatment of coronary heart disease[9], severe craniocerebral injury[10] and apoplexy[11] in China.

Pharmacokinetic studies demonstrated that after oral administration of total Panax notoginsenosides powder in rats, GRg1, GRe and NR1 reached peak concentration in plasma within 0.75 h and were eliminated quickly[8]. Thus, plasma should be collected at intensive time points to facilitate reliable pharmacokinetic analysis. However, continuous and intensive multiple time-points sampling is not applicable using the conventional serial blood sampling technique, for the volume of blood that can be withdrawn without perturbing the experimental subjects is limited. Microdialysis is an *in vivo* sampling technique that allows the measurement of endogenous and exogenous substances in the extracellular fluid surrounding the probe[12,13]. This technique has been widely applied for pharmacokinetic studies in the brain, peripheral tissues and blood. It has several advantages over conventional methods. For example, it could provide near real-time information on the time-dependent concentration changes of analytes in alive, freely moving animals such as rats and simplify the sample preparation procedure by excluding large molecules from the perfusate. The principle of the microdialysis technique is on the basis of the passive diffusion of compounds down a concentration gradient across a dialysis membrane with a specific molecular weight cutoff[14]. Thus, molecules up to a certain molar mass diffuse into (recovery) or out of (delivery) the perfusion fluid while the perfusion fluid passes the membrane, which could be used both for collecting a substance in the dialysate as well as delivering it into the periprobe fluid[15]. The latter is referred to as retrodialysis. Since the volume of the sample

obtained using microdialysis is usually in the range of 10-30 μL, an analytical method with sufficient sensitivity should be developed for the simultaneous determination of GRg1, GRe and NR1 in dialysate samples.

Several LC-MS methods have been reported for the pharmacokinetic studies of GRg1, GRe and NR1 in rat[8,16], rabbit[17] and human[18] plasma. Tedious sample preparations including protein precipitation[16,18], liquid-liquid extraction[8] and solid phase extraction[17] were employed to eliminate possible interference of the endogenous substances in plasma. However, there was no publication on the simultaneous determination of GRg1, GRe and NR1 in rat blood using microdialysis sampling technique. The purpose of this study was to establish and validate a novel sensitive LC-MS method combined with microdialysis to study the pharmacokinetic profiles of three active saponins in rat blood after intravenous administration of Xuesaitong injection.

EXPERIMENTAL

Ginsenosides Rg1, Re and notoginsenoside R1 were purchased from Jilin University (Changchun, China). Xuesaitong injection (batch number: 20081018, specification: 250 mg/10 mL) was purchased from Harbin Branch of HeiLongJiang ZBD Pharmaceutical Co., Ltd. (Harbin, China). The concentration of GRg1, GRe and NR1 in Xuesaitong were 7.55, 1.00, 2.13 mg/mL. Citric acid, sodium citrate and glucose were obtained from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Hollow fiber membrane (i.d. = 200 μm, o.d. = 280 μm) with a nominal molecular weight cut-off of 13 kDa was purchased from Spectrum Co., Ltd. (Rancho Dominguez, CA, USA). Fused silica capillary tubing (i.d. = 50 μm, o.d. = 150 μm) was obtained from Ruipu Chromatogram Equipment Co., Ltd., (Hebei, China). Polyethylene pipe-10 (i.d. = 280 μm, o.d. = 640 μm) was purchased from American Health & Medical Supply International Corp. (Chengdu, China). HPLC grade acetonitrile was purchased from Merck Company (Merck, Darmstadt, Germany). Glacial acetic acid of HPLC grade was purchased from Tedia (Fairfield, OH, USA). Other chemicals were of analytical reagent grade and purchased from commercial sources. Anticoagulant dextrose (ACD) solution was composed of 7.5 mM sodium citrate, 3.5 mM citric acid and 13.4 mM glucose. Milli-Q water (18.2 mO and TOC = 50 ppb) from Milli-Q system (Millipore SAS, Molsheim, France) was used throughout the study.

Microdialysis equipment and determination of probe recovery: Microdialysis system was composed of a CMA/100 microdialysis pump (CMA, Stockholm, Sweden), a microdialysis probe and a CMA/140 fraction collector in which the sample was collected. The microdialysis probes were made of silica capillary using a concentric design with the tips covered by a dialysis membrane as described by Tsai[19]. For the *in vitro* recovery study, microdialysis probes were immersed in the standard solution (203 μg/mL GRg1 in anticoagulant dextrose), which was maintained at 37 ºC and perfused with anticoagulant dextrose solution at 2 μL/min. The system was equilibrated for 0.5 h and then six sequential dialysate samples were collected every 10 min. A 10 μL aliquot of the dialysate sample was injected into the LC-MS system (described in the following sections) for analysis. The *in vitro* recovery was calculated as follows: $(R\ \%)_{in\ vitro} = C_d/C_s \times 100\ \%$, where C_s represented the concentration of the standard solution and C_d represented the concentration in the dialysate. The reverse dialysis or retrodialysis method was used for the determination of *in vivo* recovery. The plasma microdialysis probes were positioned within the jugular vein toward the anesthetized rats' right atrium. This measurement was performed before the doses were given to the rats. Artificial anticoagulant dextrose solution containing GRg1 (0.134 μg/mL), GRe (0.092 μg/mL) and NR1 (0.113 μg/mL) was perfused through the probe at a constant flow rate (2.0 μL/min) by the microinjection pump. After a 1 h stabilization period, six sequential dialysate samples were collected every 10 min. The *in vivo* relative recovery was calculated by the following equation:

$$R\ \% = (C_p - C_d)/C_p \times 100$$

where C_d is the analyte concentration in the dialysate and C_p that in the perfusate.

Pharmacokinetic study: Six male Sprague-Dawley rats weighing 240-260 g were provided by Laboratory Animal Center of Zhejiang University. The animal experiment was conducted in accordance with the Guidelines for Animal Experimentation of Zhejiang University (Hangzhou, China) and the procedure was approved by the Animal Ethics Committee of this institute. Prior to the experiment, the rats were fasted for 18 h with free access to water. Anesthesia was induced by the intraperitoneal (i.p.) injection of 20 % urethane (0.8 g/kg) and microdialysis probes implanted in the jugular vein of rats according to the previous developed protocol[20]. Blank anticoagulant dextrose solution was perfused at a flow rate of 2 μL/min. A dose of 10 mg/kg (0.4 mL/kg) Xuesaitong injection was administered intravenously *via* the tail vein. Microdialysis samples were collected at 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180 and 200 min. A 10 μL aliquot of the dialysate sample was immediately measured by the validated LC-MS method.

LC-MS analysis of dialysate samples: Liquid chromatography was performed on an Agilent 1100 system (Agilent, Waldbronn, Germany) consisting of an auto-sampler, diode array detector, column heater and binary pump. The chromatographic separation was achieved on an Agilent Zorbax SB-C_{18} column (2.1 mm × 100 mm i.d., 3.5 μm, Agilent, Wilmington, DE, USA) and the column temperature was maintained at 30 ºC. A mixture of water containing 0.05 vol % acetic acid (A) and acetonitrile with 0.05 vol % acetic acid (B) was used as a mobile phase with the flow rate 0.25 mL/min. The separation was finished within 10 min using linear gradient elution from 23 to 80 % (v/v) B. Mass detection was operated on an Agilent 1100 single-quadrupole mass spectrometer (G1946D) at negative selective ion monitoring (SIM) mode using an electrospray ionization source. The typical source conditions were as follows: flow-rate of drying gas, 9 L/min, source temperature 350 ºC, nebulizer pressure (N_2) 35 psi and capillary voltage 5.0 kV. The ions at m/z 859 ($[M + CH_3COO]^-$), 931 ($[M-H]^-$), 945 ($[M-H]^-$) were selected for the quantification of GRg1, NR1 and GRe, respectively. The corresponding fragmentor voltages were 200, 350 and 350 V. The microdialysis samples in the present study were analyzed without prior sample purification.

A 10 µL aliquot of the sample was injected into the LC-ESI-MS system.

Standard stock solutions of GRg1, GRe and NR1 were prepared individually in methanol at the concentrations 1.185, 1.050 and 1.160 mg/mL, respectively. Serial working solutions were prepared by further dilutions of the corresponding stock solution with anticoagulant dextrose solution to obtain the desired concentrations of 0.01185, 0.05925, 0.1185, 0.5925, 1.185, 5.925 and 11.85 µg/mL for GRg1, 0.0105, 0.0525, 0.105, 0.525, 1.05, 5.25 and 10.5 µg/mL for GRe and 0.0116, 0.058, 0.116, 0.580, 1.16, 5.80 and 11.6 µg/mL for NR1, respectively. All the solutions were stored at 4 ºC and brought to room temperature before use. In order to evaluate the specificity of the analytical method, drug-free matrices, artificial microdialyzate and authentic samples from Sprague-Dawley rats were investigated for compounds influencing analysis. Peak areas of endogenous compounds co-eluting with the analytes should be less than 20 % of the peak area of the LLOQ standard according to international guidelines[21]. Linearity was evaluated by analyzing seven spiked calibration samples and the calibration curve was constructed using an external standard method. The acceptance criterion for each back-calculated standard concentration was 15 % deviation from the nominal value[21]. The intra-day and inter-day variability were determined by assessing the variance in six individual samples on the same day and six successive days, respectively. The assay accuracy was expressed as relative error (RE), *i.e.* (observed concentration-nominal concentration)/(nominal concentration) × 100 %. The assay precision was calculated by using the relative standard deviation (RSD). The intra-day and inter-day precisions were required to be below 15 % and the accuracy to be within ± 15 %[21]. The stability of analytes in microdialysate was assessed by analyzing quality control samples kept for 24 h and 6 days at room temperature. All stability samples at three concentration levels (GRg1: 0.029625, 1.185 and 5.925 µg/mL; NR1: 0.0290, 1.16 and 5.800 µg/mL; GRe: 0.02625, 1.05 and 5.250 µg/mL) were analyzed and the deviations were determined in relation to freshly prepared samples. The analytes are considered to be stable when the precisions are below 15 % and the accuracies are in the range of 85-115 % respectively for three levels.

Data analysis: Different to blood sampling, the concentration values in microdialysis samples (corrected by recovery) represent mean values, the integral of the concentration surrounding the probe, during the sampling time period. Up to now, no commercial softwares have been designed to analyze such data directly. The sampling times should be corrected by an iterative algorithm[22] or just replaced by the midpoint of sampling time[23] before pharmacokinetic parameters could be calculated. The complexity of the iterative algorithm hindered its application in bioanalytical fields and most researchers would prefer the imprecise midpoint method. To circumvent the mentioned problems of microdialysis data analysis, a user-defined model was developed using WinNonLin (v6.1, Pharsight, Mountain View, CA, USA) (supplied in appendix). In this model, the vector of cumulative area under the curve (AUC_i, i = 1…n) was fitted to the time vector (t_i, i = 1…n) and AUC_i obtained from the following equation:

$$AUC_i = C_1 \times t_1 + ... + C_i \times t_i$$

where C_i (i = 1..n) was the recovery corrected concentration vector of microdialysis samples. So the differentiate of AUC (line 13 in appendix) was the real concentration (Cr) surrounding the probe. Since the concentration (Cr) of any compartmental models could be obtained either by algebraic equations or differential equations[24], all pharmacokinetic data could be analyzed by this model in theory. In the current case, the two differential equations at lines 11 and 12 in appendix were used to represent a two compartment model. The calculation of other pharmacokinetic parameters was the same as classical models as expressed in the appendix.

RESULTS AND DISCUSSION

Evaluation of the microdialysis set-up: For the validity of the experimental outcomes, several factors should be considered about the microdialysis set-up. Firstly, the external concentrations (101, 203 and 406 µg/mL of GRg1 in anticoagulant dextrose solutions) and perfusion flow rates (2.0, 4.0 and 8.0 µL/min) on the impact of *in vitro* probe recovery were investigated. As listed in Table-1, the recovery decreased with the increase of perfusion flow rates and different concentration levels showed the same trend. Moreover, significant variation ($p < 0.05$) of recoveries were observed at high flow rates for different concentration levels. Therefore, high perfusion flow rate should be avoided while performing the microdialysis experiment. Although high recovery and high stability could be achieved when the perfusion flow rate was lower than 2 µL/min, the sampling time would be sacrificed. Then, the permeability of the probe before and after 12 h implantation in rats was compared. It showed no significant difference between the pre- and post- *in vitro* recoveries. This indicated that this homemade probe was stable after *in vivo* sampling for 12 h. Because *in vitro* recovery may significantly differ from the *in vivo* recovery[23], it was important to measure *in vivo* recovery to converse microdialyzate concentrations into extracellular concentrations. In present experiment, average *in vivo* recoveries (n = 6) of GRg1, GRe and NR1 were 7.35 ± 1.58, 5.11 ± 1.75 and 4.80 ± 1.25, respectively.

TABLE-1
In vitro RECOVERY ESTIMATED UNDER DIFFERENT EXTERNAL CONCENTRATIONS OF GRg1 AND PERFUSION FLOW RATES (MEAN ± SD)

External concentration	*In vitro* recovery (%)		
(µg/mL)	2 µL/min	4 µL/min	8 µL/min
101	11.8 ± 0.28	5.59 ± 0.37	2.44 ± 0.15*
203	11.8 ± 0.94	6.24 ± 0.72*	1.40 ± 0.25
406	12.6 ± 0.62	5.78 ± 0.50	1.16 ± 0.50

*Significant difference in comparison with others ($p < 0.05$)

LC/MS method validation: Fig. 1 showed the optimized chromatograms of a blank dialyzate sample, an anticoagulant dextrose solution spiked with the corresponding standard solution at LLOQ and a rat plasma dialyzate sample obtained at 25 min after intravenous administration of 10 mg/kg Xuesaitong injection. No significant endogenous interferences were observed at the retention times of the analytes. This analytical method thus exhibited excellent selectivity in determination of GRg1, GRe and NR1 in microdialyzate samples. The method exhibited excellent linear response over the selected concentration range

(GRg1: 0.01185 - 11.85 µg/mL, GRe: 0.0105 - 10.5 µg/mL, NR1: 0.0116 - 11.6 µg/mL) by linear regression analysis. The correlation coefficient (r^2) was > 0.9958 for all the three analytes. LLOQ for GRg1, GRe and NR1 was 0.01185, 0.0105 and 0.0116 µg/mL, respectively, which was sensitive enough for the present pharmacokinetic study. Table-2 presents the accuracy and intra and inter assay precision, which was determined by analyzing six replicates of quality control samples at three concentrations on six different days. The intra and inter assay precisions were measured to be below 5.34 and 6.15 %, respectively. The accuracy of the method, expressed in terms of RE, ranged from -2.8 to 7.5 % at three quality control levels. The above results indicated that the values were within the acceptable range and the method was accurate and precise. Stability results indicated that the analytes in microdialysate were stable at room temperature for 24 h and 6 days.

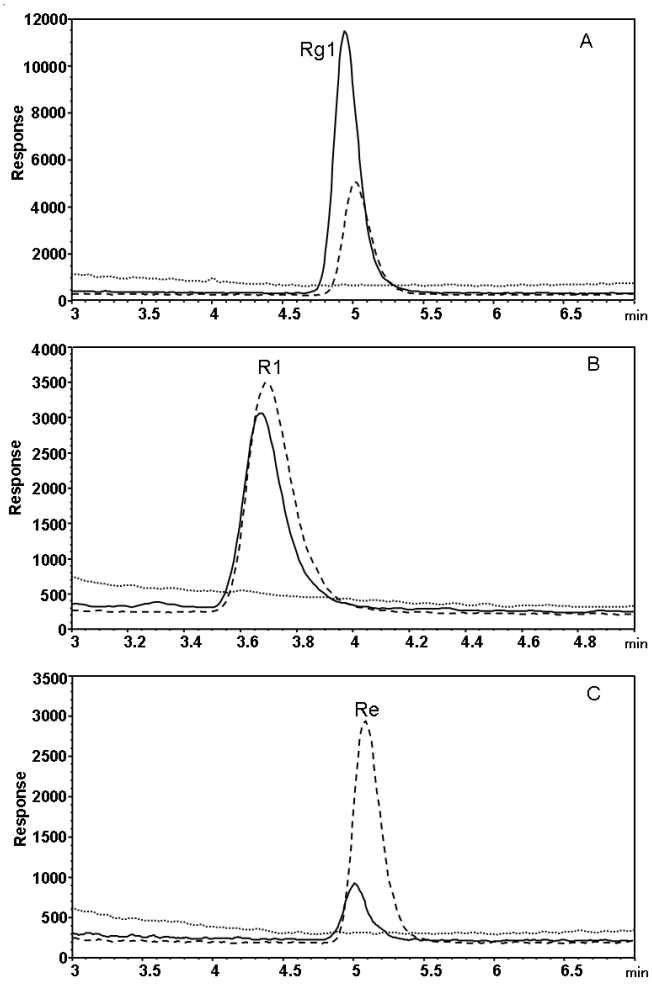

Fig. 1. Typical chromatograms of ginsenoside Rg1 (A), notoginsenoside R1 (B) and ginsenoside Re (C) registered by selective ion monitoring scan mode, where dotted lines represent an analyte-free microdialysate, solid lines represent a rat microdialysate sample obtained at 25 min after intravenous administration of 10 mg/kg Xuesaitong injection and dash lines represent an analyte-free microdialysate spiked with ginsenoside Rg1, notoginsenoside R1 and ginsenoside Re at concentrations of 0.01185, 0.0116 and 0.0105 µg/mL, respectively

Pharmacokinetic evaluation of Xuesaitong injection in rats: The microdialysis-LC-MS method described above had been applied successfully to the pharmacokinetic study of GRg1, GRe and NR1 in Sprague-Dawley rats. As shown in Fig. 2, the high density sampling, especially for the first hour, of microdialysis have enabled the characterization of the fast elimination of GRg1, GRe and NR1. Their corresponding pharmacokinetic parameters (Table-3) were calculated from the relationship of cumulative area under the curve (AUC) and time by applying the user-defined WinNonLin model, in which only one more derivative equation (dAUC/dt = C), the relationship of AUC and concentration, was added when comparing to normal compartment models. Thus, the sampling time values do not need to be corrected, simple and direct for bioanalytical end-users. The AUC *versus* time profiles (solid

TABLE-2
INTRA ASSAY AND INTER ASSAY PRECISION (RSD) AND ACCURACY (RE) OF THE LC-MS METHOD FOR THE DETERMINATION OF GRg1, GRe AND NR1.

Compound	Concentration (µg/mL)		RSD (%)		RE (%)
	Added	Found	Intra-run	Inter-run	
GRg1	0.029	0.030	5.34	3.84	1.8
	1.185	1.247	2.86	6.15	5.2
	5.925	6.180	1.53	5.21	4.3
NR1	0.029	0.028	2.51	2.86	-1.6
	1.160	1.142	1.11	4.03	-1.6
	5.800	6.235	2.15	3.37	7.5
GRe	0.026	0.027	3.12	2.92	2.9
	1.050	1.021	1.62	3.78	-2.8
	5.250	5.581	2.19	3.27	6.3

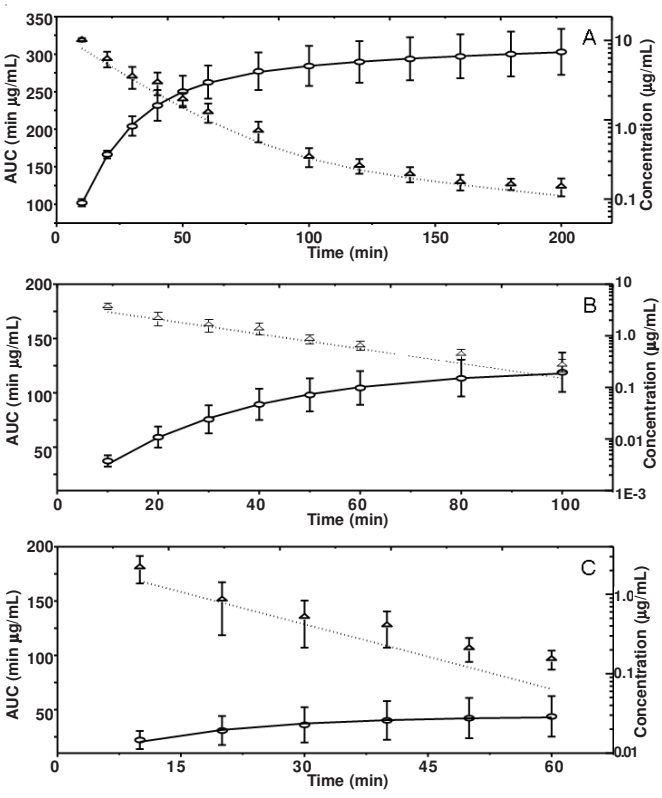

Fig. 2. AUC *versus* time (left Y-axis, O) and blood concentration *versus* time (right Y-axis, Δ) profiles of ginsenoside Rg1 (A), notoginsenoside R1 (B) and ginsenoside Re (C) after intravenous administration of 10 mg/kg Xuesaitong injection (mean ± SD), where solid lines represent the simulated AUC values and their corresponding simulated concentration values are given by dotted lines

TABLE-3
PHARMACOKINETIC PARAMETERS OF GRg1, GRe AND NR1 AFTER INTRAVENOUS ADMINISTRATION OF 10 mg/kg XUESAITONG INJECTION TO SIX MALE SD RATS (MEAN ± SD)

Parameters	GRg1	NR1	GRe
K10 (1/h)	2.39 ± 0.090	1.97 ± 0.062	3.76 ± 0.287
K12 (1/h)	0.41 ± 0.048	–	–
K21 (1/h)	0.53 ± 0.212	–	–
V1 (L/kg)	0.24 ± 0.002	0.21 ± 0.005	0.15 ± 0.009
AUC (h*μg/mL)	5.29 ± 0.162	2.04 ± 0.025	0.73 ± 0.017
K10_HL (h)	0.29 ± 0.011	0.35 ± 0.011	0.18 ± 0.014
ALPHA_HL (h)	0.24 ± 0.007	–	–
BETA_HL (h)	1.59 ± 0.653	–	–
ALPHA (1/h)	2.89 ± 0.090	–	–
BETA (1/h)	0.44 ± 0.179	–	–
CL (L/h/kg)	0.57 ± 0.017	0.42 ± 0.005	0.56 ± 0.013
AUMC (h*h*μg/mL)	3.92 ± 1.126	1.03 ± 0.044	0.20 ± 0.019
MRT (h)	0.74 ± 0.190	0.51 ± 0.016	0.27 ± 0.020
V2 (L/kg)	0.18 ± 0.094	–	–

lines in Fig. 2) were fitted well by one or two compartment models. Then, the real concentration profiles (dotted lines in Fig. 2) in blood were simulated using the obtained pharmacokinetic parameters. As expected, all the recovery corrected concentrations were above the simulated lines. The concentration-time profile of GRg1 demonstrated a two-phase character; only one phase was observed for NR1 and GRe because most of the sample concentrations after 1 h were under the detection limit. Even though, their first phase half lives (ALPHA_HL for GRg1, K10_HL for GR1 and GRe) were very near (0.18-0.35 h), suggesting similar pharmaco-kinetic characteristics for these three saponins. The systemic clearances of GRg1, GRe and NR1 were 0.57, 0.56 and 0.42 L/h/kg at a 10 mg/kg dose, respectively. Statistical analysis revealed no significant difference among systemic clearances values for the three saponins. These results were not consistent with the reported values[8,25] (1.91, 10.72 and 5.22 L/h/kg for GRg1, GRe and NR1 at the same dose level). It seemed that the total doses (10 mg/kg) other than the real doses of GRg1, GRe and NR1 (3.02, 0.40, 0.85 mg/kg) were utilized for their calculation; the dose-corrected values of Li *et al.* were 0.57, 0.43, 0.44 L/h/kg. Collectively, the developed microdialysis-LC-MS method could characterize the rapid disposition of GRg1, GRe and NR1 in rats.

Conclusion

A selective and highly efficient method by microdialysis coupling to LC-MS for the determination of GRg1, GRe and NR1 in rats has been developed and validated. With respect to the previous blood sampling method, the present method had the advantages of high temporal resolution, no biological fluid loss and no endogenous interference. It is capable for characterization of fast eliminating drugs, long-peroid continuous sampling and more, simplifying the sample pretreatment procedures. The pharmacokinetic profiles of GRg1, GRe and NR1 were studied by this method after intravenous administration of 10 mg/kg Xuesaitong injection to rats. Main pharmacokinetic parameters were estimated using the industry standard software - WinNonLin by developing a user-defined model. The precision of parameters was improved and comparable results obtained by this new model. This was a new attempt to analyze *in vivo* microdialysis data directly and would be a good example for the pharmacokinetic application of microdialysis technology.

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