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Short communication

Ultra-performance liquid chromatography–tandem mass spectrometric method for the determination of Artemisinin in rat serum and its application in pharmacokinetics

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Abstract

A rapid and sensitive ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method was developed and validated to quantify artemisinin in rat serum. The lower limit of quantification (LLOQ) was 4 ng/mL. The calibration curve was linear from 4 ng/mL to 10,000 ng/mL ($R = 0.998$). The assay was based on the selected reaction monitoring (SRM) transitions at *m/z* 305.4–151.10 for artemisinin and *m/z* 335.2–163.10 for arteether (internal standard). The artemisinin and internal standard can be separated from endogenous interferences in rat serum. Inter- and intra-day assay variation was less than 15%. The extraction recoveries ranged from 80.0 to 107.3% at the three concentrations (5000, 2000, and 200 ng/mL). This method was successfully applied to pharmacokinetic studies of artemisinin after intravenous and oral administration to rats.

Keywords: Artemisinin; UPLC; MS/MS; Pharmacokinetic; Rat serum

1. Introduction

Malaria is still a major public health threat, with over one billion people at risk of contracting this deadly disease. Among all of the antimalarial agents on the market, artemisinin and its semisynthetic family of derivatives are the most potent antimalarials available for the treatment of *Plasmodium falciparum* infections [1]. Artemisinin is a sesquiterpene-lactone endoperoxide isolated from *Artemisia annua* L., a plant with a long history of medical use against malaria in China. This compound and its derivatives are effective against both chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum*, as well as cerebral malaria [2,3].

To further understand and reveal the pharmacokinetic profile and action of artemisinin and its derivatives, sensitive and specific analytical methods are needed. In the past 20 years, many analytical techniques have been reported for the qualitative and quantitative determination of artemisinin and its derivatives in biological matrices. High-performance liquid chromatography with UV or electrochemical detection has been used for the quantification of artemisinin [4–16]. Kuroda et al. [7] used HPLC with an on-line photoreactor and peroxyoxalate chemiluminescence detection to determine artemisinin in biological fluids. Kohler et al. [17] used off-line supercritical fluid extraction and supercritical fluid chromatography coupled to an evaporative light-scattering detector to determine artemisinin and artemisinic acid in plant extracts. TLC, HP-TLC and GC methods were also developed and optimized for determination of artemisinin [18–21]. Each method had limitations. Some HPLC-UV methods required lengthy derivatization procedures prior to sample preparation. Some lacked the required sensitivity to be used for measurement in biological samples. Liquid chromatography (LC) coupled with electrochemical detection (EC) was reported to be tedious and cannot be applied in routine analysis [22]. More important fact is that most of these methods lack selectivity. Liquid chromatography coupled to tandem

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mass spectrometry is known to be a powerful separation and detection technique in a large number of analytical fields. Studies have been reported using a successful quantification method for artemisinin in biological fluids or plant extracts by LC/MS or LC-MS/MS [23–27]. Maillard et al. reported early LC-MS method for the analysis of artemisinin in crude plant extracts [26]. Wang et al. [23] and Saihai et al. [24] further developed LC-MS method with selective ion monitoring (SIM) mode to determine artemisinin in plant extracts. These three methods choose to use (SIM), which is usually not as specific or sensitive as selected reaction monitoring (SRM). Xing et al. [25] and Nieuwerburgh et al. [27] developed LC-MS/MS method with SRM mode to detect artemisinin in rat plasma and plant extract, which was reported to have the excellent specificity. However, the retention time of artemisinin was found to be 6 and 10 min in these methods. It is still not fast enough for high throughput sample processing.

More recently, the search for a method with a significantly reduced analysis time and increased sample throughput, sensitivity and resolution has resulted in the development of a new technology termed ultra-performance liquid chromatography (UPLC). This paper presents the development and validation of an assay method for the determination and quantification of artemisinin in rat serum by UPLC-MS/MS, using an ESI interface operated in positive mode. In this method, high sample throughput is achieved by simple sample preparation and short chromatographic run times under isocratic conditions. As we know, a problem in pharmacokinetic studies with artemisinin and its analogues is the fact that their absolute bioavailabilities are difficult to calculate because of the lack of an intravenous formulation [28]. This analytical method was successfully applied to pharmacokinetic studies of artemisinin in rats and the pharmacokinetic parameters of artemisinin after intravenous administration to rats will be useful for future bioavailability study of artemisinin and its analogues.

2. Experimental

2.1. Chemicals and reagents

Reference standards of artemisinin and β-arteether were obtained from Sigma–Aldrich (Milwaukee, WI, USA); HPLC grade acetonitrile, methanol and water were from Fisher (Fair lawn, NJ, USA); Reagent grade formic acid was purchased from Sigma–Aldrich (95%, St. Louis, MO, USA); Drug-free rat serum was bought from Innovative Research Inc. (Southfield, MI, USA).

2.2. Liquid chromatography

A Waters Acquity UPLC system was used for method development of artemisinin (Milford, MA, USA). Chromatographic separations were performed on a Waters Acquity UPLC^{TM} BEH C_{18} column (1.7 μm, 2.1 mm × 50 mm). The mobile phase consisted of methanol:0.3% formic acid (80:20, v/v). The flow rate was 0.3 ml/min, the sample injection volume was 15 μl, and the duration of the run was 3 min.

2.3. Mass spectrometry

The MS instrument consisted of a Waters Micromass Quattro Micro^{TM} triple-quadrupole system (Manchester, UK). The MS system was controlled by version 4.0 of MassLynx software. Ionization was performed in the positive electrospray mode. MS/MS conditions were the following: capillary voltage 3.5 kV, cone voltage 40 V, extractor voltage 2 V, RF lens voltage 0.2 V. The source and desolvation temperatures were 100 and 350 °C, respectively, and the desolvation and cone gas flows were 550 and 25 L/h, respectively.

The selected mass-to-charge (*m/z*) ratio transitions of the artemisinin and arteether ions $[M+Na]^+$ used in the SRM were as follows: artemisinin, *m/z* 305.4–151.10, arteether, *m/z* 335.2–163.10. The collision energy for fragmentation of the precursor ions was set at 22 and 21 eV for artemisinin and internal standard, respectively. The dwell time was set at 500 ms.

2.4. Standard and working solutions

Individual standard stock solutions of artemisinin (1 mg/mL) and the internal standard (20 μg/mL) were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in methanol.

The calibration standard for artemisinin was prepared by spiking 5 mL of blank rat serum with 50 μL of artemisinin stock solution. The resulting serum standard had a concentration of 10,000 ng/mL. Further dilutions were made from this stock with blank serum to afford serum standards in the range of 4–10,000 ng/mL. Quality control (QC) samples at four different concentrations of 80, 500, 3000, and 5000 ng/mL were prepared separately. The QC samples were used to assess the accuracy and precision of the assay method. All the calibration and QC samples were then extracted by the method described in the subsequent section and analyzed. The QC samples were stored along with the test samples at −30 °C until analysis.

2.5. Sample preparation

A mixture consisting of 100 μL of rat serum and a 50 μL aliquot of internal standard solution was placed in an Eppendorf micro tube for processing. Acetonitrile (300 μL) was added to precipitate proteins, and the micro tube was vortexed for 5 min and centrifuged for 20 min at 10,000 rpm (Eppendorf 5415C centrifuge). The supernatant was filtered with a 0.2 μm filter (Waters 13 mm GHP 0.2 μm) before analysis.

2.6. Oral and intravenous formulation

The oral formulation of artemisinin was prepared as an aqueous suspension in 1 mL 10% DMSO/0.05% Tween 80 solution. The oral formulation was administered to rats at a dose of 100 mg/kg. Any drug-related materials remaining in the tube or syringe after dosing were dissolved in a known volume of acetonitrile and assayed by LC-MS/MS to enable determination of the exact dose administered.

An aqueous solution comprising 25% DMSO/10% Cremophore EL was selected as it enabled intravenous administration of artemisinin at an 8 mg/kg dose. The intravenous formulation was prepared on the day of dosing and sterile filtered through a 0.2 μm syringe filter immediately prior to use.

2.7. Animal study

All the experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi. The Sprague Dawley rats (290–330 g) were obtained from the Harlan Company (Indianapolis, IN) and already had a polyethylene cannula inserted into the right jugular vein. The rats were housed in standard cages and allowed free movement and access to food and water during the whole experiment.

The IV solution was administered via the jugular vein cannula, after which the cannula was flushed with 0.2 ml heparinised saline to ensure complete administration of the dose. The oral formulation was gavage dosed to the rats. The tube containing the suspension was then rinsed with 1 mL water, which was also then administered to the rat to ensure complete dosing.

Blood samples were taken from the indwelling cannula. An initial blood volume of 0.1 mL was withdrawn to clear the line of heparinised saline. A fresh syringe was then used to withdraw a 0.2 mL blood sample which was placed in a micro tube. The blood samples were allowed to clot at room temperature for over 30 min. Serum was separated by centrifugation at 3000 rpm for 10 min and was stored at −30 °C.

2.8. Method validation

The validation of the LC-MS/MS method included linearity, selectivity, inter- and intra-assay precision and accuracy, stability, recovery and ion suppression studies.

2.8.1. Selectivity

Pool of six blank serum samples was extracted and analyzed to assure that it is free of interfering response values. The apparent response at the retention time of artemisinin and internal standard were compared to the response at the limit of quantitation.

2.8.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were calculated based on the relationship between the ratio of the peak area of artemisinin to that of the internal standard and the theoretical concentration of analyte. The calibration was processed with MassLynx 4.0 QuanLynx software. The lower limit of quantification was defined as the concentration of the artemisinin at which the response of artemisinin is ten times the response compared to the blank response.

2.8.3. Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of the four QC samples with five determinations per concentration in the same day. The inter-day accuracy and precision was measured over three days. Precision was measured by inter- and intra-assay % R.S.D. The accuracy was evaluated by the deviation or bias (%) of the observed concentration from the actual concentration.

2.8.4. Recovery and matrix effects

The recovery of the method was obtained in triplicate at three final concentration levels (5000, 2000, and 200 ng/mL) from a detector response of the analyte added to and extracted from the serum, compared to the detector response of the analyte spiked after extraction into serum extracts [29].

The matrix effect of rat serum can be examined by comparing the MS/MS response of the analyte at three concentrations spiked post-extraction into serum to the MS/MS response of the same analyte present in the neat mobile phase [29].

2.8.5. Freeze-thaw and short-term stabilities

The QC samples at four final concentrations (5000, 2500, 500, and 80 ng/mL) were stored at −30 °C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 12–24 h under the same conditions. After three cycles, the percent loss of the analyte was determined by comparing the concentrations with those obtained before freezing.

For the short-term stability test, the QC samples at the same four concentrations were thawed at room temperature and kept at this temperature from 4 to 24 h and analyzed.

2.8.6. Application of the method to biological samples

The assay method described here was applied to study the pharmacokinetic in rat serum after intravenous artemisinin to rats. The pharmacokinetic parameters were calculated by WinNonlin professional software version 5.0.1 (Pharsight, Mountain View, CA, USA) using the noncompartment model. Peak plasma concentration (C_{max}) was obtained from observed data. Plasma $AUC_{0\rightarrow t}$ values (t being the time of the last plasma concentration measured) were calculated by the linear trapezoidal rule. The first order rate constant, λz, was estimated by linear regression of time versus log of the concentration. Plasma clearance (CL_p), terminal half-life ($t_{1/2}$), and apparent volume of distribution (V_d) were also calculated.

3. Result and discussion

3.1. Ultra-performance liquid chromatography and sample preparation

High-performance liquid chromatography has been widely used for the analysis of artemisinin in biological samples. Recently, an improvement in chromatographic performance has been achieved by the introduction of UPLC. The van Deemter equation indicates that, as the particle size decreases to less than 2.5 μm, there is a significant gain in efficiency and that efficiency does not diminish at increased flow rates or linear velocities. Thus, UPLC takes full advantage of chromatographic principles to run separations using columns packed with smaller

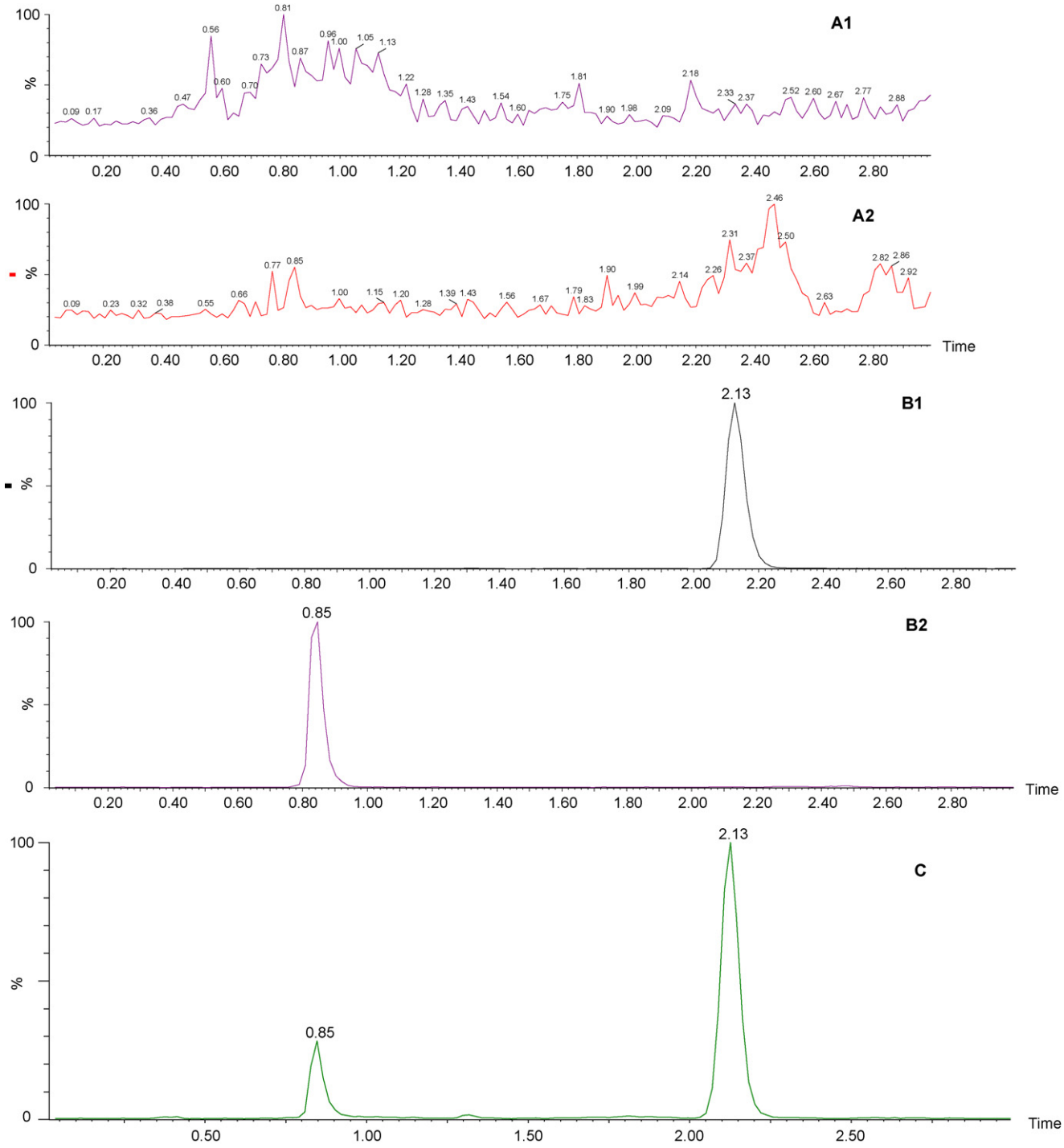

Fig. 1. (A) Chromatogram for blank serum at *m/z* 335.2–163.10 (for internal standard, A1) and *m/z* 305.4–151.10 (for artemisinin, A2). (B) Chromatogram for blank serum spiked with 2000 ng/mL artemisinin (B2) and 100 ng/mL I.S. (B1). (C) Chromatogram for serum samples 15 min after intravenous administration spiked with internal standard at *m/z* 335.2–163.1 and *m/z* 305.4–151.1 mode.

particles (1.7 μm), with superior resolution and shorter analysis time [30,31].

In our experiment, a 100 mm column submitted to an isocratic flow rate of 0.3 mL/min for 3 min was used to obtain the chromatograms. The very narrow chromatographic peaks generated by UPLC, with peak width lower than 2s, resulted in an increase in the chromatographic efficiency and sensitivity.

The pretreatment of the serum samples proved to be very suitable for a protein-rich biological matrix. Acetonitrile was used to precipitate proteins. Clear solutions were obtained, and the supernatant was easily recovered by simply decanting the upper liquid from the precipitated proteins. An extra advantage of this approach was the possibility of simultaneously processing a large number of samples. No evaporation of the sample

Table 1
Summary of the accuracy and precision of artemisinin in rat serum

Spiked concentration (ng/ml)	Intra-day precision and accuracy ($n=4$)			Inter-day precision and accuracy ($n=3$)		
	Measured concentration (ng/ml)	R.S.D. (%)	Mean recovery (%) ± S.D.	Measured concentration (ng/ml)	R.S.D. (%)	Mean recovery (%) ± S.D.
5000	5591.8 ± 885.7	15.8	111.8 ± 17.7	5378.9 ± 373.6	6.9	107.6 ± 7.5
3000	3751.0 ± 95.9	2.6	125.0 ± 3.2	3586.6 ± 326.5	9.1	119.6 ± 10.9
800	952.1 ± 62.2	6.5	119.0 ± 7.8	865.7 ± 83.7	9.7	108.2 ± 10.5
80	77.6 ± 5.8	7.5	96.9 ± 7.3	68.0 ± 4.6	6.8	85.0 ± 5.8

R.S.D., relative standard deviation; recovery = 1-(nominal concentration-measured concentration)/nominal concentration.

to dryness was needed for this procedure that can decrease partial loss of the analyte due to possible thermal degradation or adsorptive loss.

3.2. Mass spectrometry

The advantages supplied by the UPLC system were strengthened with its coupling to a tandem quadrupole mass spectrometer, which provided enhanced selectivity and sensitivity. The optimization of MS/MS detection was carried out in the electrospray positive ion mode. The mobile phase optimization was done by comparing various solvent systems composed by mixtures of methanol or acetonitrile with formic acid, acetic acid, ammonium acetate and ammonium hydroxide solutions. The mobile phase consisting of a mixture of methanol:0.3% formic acid was found to be suitable for separation and ionization of artemisininin and arteether. The selection of SRM transitions and associated acquisition parameters (collision energy and cone voltage) was evaluated for best response under positive mode by infusing a standard solution via a syringe pump into the mobile phase. The optimized responses for artemisinin and arteether were obtained. For artemisinin several possible daughter ions can be considered (*m/z* 151.1, *m/z* 194.1 and *m/z* 263.4). The fragment at *m/z* 151.1 was selected because it was the most abundant daughter ion obtained compared to other daughter ions. Protonated species $[M + Na^+]$ and their respective daughter ions were monitored at *m/z* 315.4 > 151.1 for artemisinin, and *m/z* 335.2 > 163.1 for arteether.

3.3. Selectivity

UPLC-MS/MS analysis of the serum samples showed no endogenous peak interference with the quantification of artemisinin and the internal standard. Representative chromatograms of blank rat serum spiked with artemisinin and internal standard, blank serum and extracted serum samples are shown in Fig. 1. No artemisinin was detected in the serum samples after oral administration with this method.

3.4. Linearity and lower limit of quantification

The calibration model was selected based on the analysis of the data by linear regression with intercepts and 1/× weighting factor. The linear equation was $Y = 8.91e-5X + 1.12e-4$. Each standard point was back calculated with the calibration. The no-zero standards showed less than 20% deviation at the LLOQ and less than 15% deviation at all other concentration levels. The coefficient (r) for artemisinin was 0.9981. The calibration range was selected according to the concentrations anticipated in the samples to be determined. The final calibration range was 4–10,000 ng/mL. The lower limit of quantification was 4 ng/mL.

3.5. Accuracy and precision

The results of the accuracy and precision at the four concentrations are presented in Table 1. The repeatability and reproducibility bias (%) is within the acceptance limits of ±20% at low concentration and ±15% at other concentration levels. The results also show that the analytical method is accurate with recoveries in the range of 84.5–114.0%.

3.6. Recovery and matrix effect

The mean recoveries of this method were 107.3 ± 1.5% at 5000 ng/ml, 99.2 ± 2.7% at 2000 ng/ml and 80.0 ± 1.8% at 200 ng/ml. These results suggested that there was no relevant difference in extraction recovery at different concentration levels.

Matrix effect is defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample. It could be a severe problem when developing LC-MS assays. In our experiment, the matrix effect of the studied serum was calculated by the ratio of the response of spiked samples after extraction over the response of the corresponding acetonitrile samples. The matrix effect was found to be 65.0 ± 5% for artemisinin. The chromatographic parameters were optimized in such a way that the analyte and the internal standard were eluted at different time. The absolute peak area of the arteether was used as a marker to monitor possible suppression effects with study samples. The R.S.D. of the arteether peak area was found to be less than 20% for batches of more than 150 samples including samples from different subjects, calibration and QC samples.

3.7. Stability

The results of freeze-thaw and short-term storage stability are shown in Table 2. The artemisinin was found to be stable after three freeze-thaw circles at four different concentrations. Artemisinin in rat serum at four concentrations stored in room

Table 2
Results of short-term and freeze-thaw stability studies of artemisinin in rat serum

Stability	Time (h)/ circle (times)	Concentration (ng/mL)							
		5000		2500		500		80	
		Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)	Recovery (%) ± S.D.	R.S.D. (%)
Short-time stability	4	71.8 ± 6.0	8.3	76.4 ± 0.6	0.8	70.7 ± 2.3	3.3	107.2 ± 5.2	4.9
	6	73.8 ± 11.2	15.2	76.4 ± 5.6	7.3	74.2 ± 11.0	14.8	92.8 ± 2.2	2.4
	12	49.0 ± 1.7	3.5	45.4 ± 1.8	4.0	64.1 ± 1.0	1.6	88.7 ± 4.9	5.5
Freeze-thaw stability	1	87.5 ± 2.6	3.0	75.1 ± 3.2	4.3	73.5 ± 2.6	3.5	113.8 ± 9.4	8.3
	2	71.5 ± 2.1	2.9	79.6 ± 2.6	0.5	73.2 ± 2.7	3.7	118.5 ± 8.3	7.0
	3	75.1 ± 2.5	3.3	79.2 ± 1.9	2.4	73.8 ± 3.7	5.0	115.7 ± 7.9	6.8

R.S.D., relative standard deviation.

temperature were found to be stable at least for 6 h. The mean recoveries from the nominal concentrations were between 70 and 100% at three different concentrations. Extracts of the QC samples were found to be stable on the auto-sampler at room temperature for at least 12 h.

3.8. Optimization of the intravenous formulation

Even though the oral formulations of artemisinin and its analogues are optimized by pharmaceutical scientists [28,32–35], there is no intravenous formulation of artemisinin available in the market till now. Our aim was to find a formulation which would increase the solubility of artemisnin and decrease its precipitation in blood. As we know, cosolvents are water-miscible organic solvents which are used in liquid drug formulation to increase the solubility of poorly water-soluble substances or enhance the chemical stability of a drug [36]. In many cases, it was proved that the use of cosolvents in the preparation of formulations for nonpolar drugs is a simple and potentially effective way to achieve high concentration of drugs. Several cosolvents were selected as vehicles for intravenous formulation of artemisinin such as PEG400, PEG300, propylene glycol, glycerin, ethanol, DMSO and Tween80. DMSO with Cremophor EL as surfactant was found to greatly increase the solubility of artemisinin and decrease the possibility of precipitation. The final blank intravenous formulation was administered to rats and the rats were found to be normal during the whole experiment.

3.9. Application to the pharmacokinetic study

Fig. 2 shows the plasma concentration versus time profiles for artemisinin in rat after intravenous administration. The concentration of artemisinin decreased quickly after intravenous administration; then the concentration maintained at a relative stable level for several hours. Noncompartment model was used to calculate the pharmacokinetic parameters with WinNonlin software. The corresponding results of the pharmacokinetic parameters are shown in Table 3. The PK data analysis showed that artemisinin has moderate clearance and a large volume of distribution. This result will be good reference for future absolute bioavailability study.

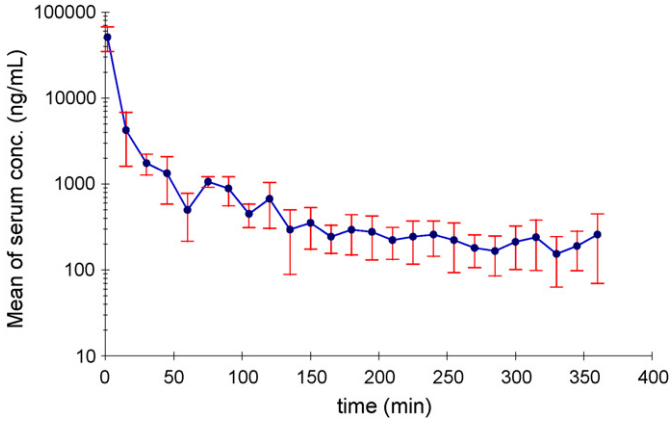

Fig. 2. Mean concentrations of artemisinin in serum after i.v. administration versus time profile ($n = 3$).

Table 3
Estimated pharmacokinetic parameters after intravenous administration of artemisinin (8 mg/kg) to rats (three rats per sampling time points)

Parameter	Unit	Mean ± S.D.	S.E.
C_{max}	ng/mL	51,127.83 ± 28,045.83	12,683.38
$t_{1/2}$	min	101.23 ± 36.71	21.19
V_d	mL/kg	1898.31 ± 635.15	366.7
CL_p	mL/min/kg	15.59 ± 11.61	6.71
$AUC_{0\rightarrow t}$	min ng/mL	647,999.41 ± 346,895.04	200,279.94
λ_z	1/min	0.0076 ± 0.0033	0.0019

C_{max}, peak plasma concentration; $t_{1/2}$, terminal elimination half life; AUC, area under the plasma concentration-time curve; V_d, apparent volume of distribution; CL_p, plasma clearance; λ_z, first order rate constant; S.D., standard deviation; S.E., standard error.

4. Conclusion

The pharmacokinetic analysis of artemisinin relies on a highly sensitive assay, capable of determining artemisinin in serum at a wide concentration range after intravenous administration. The limited volumes of serum and interferences from the biological matrix all added to the complexity of the trace analysis of artemisinin. The UPLC-MS/MS assay reported here has demonstrated a rapid separation and specific identification of artemisinin from rat serum. The assay uses β-arteether as the internal standard. The extraction procedure is simple and rela-

tively short, allowing sufficient sample-throughput to be applied to pharmacokinetic studies of artemisinin. The results of the validation show that the method is reproducible and accurate. The analysis requires only 100 μL serum which is advantageous in pharmacokinetic studies. Thus, the method proved to be applicable for further pharmacokinetic studies.

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