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Determination of antimalarial compound, ARB-89 (7β-hydroxy-artemisinin carbamate) in rat serum by UPLC/MS/MS and its application in pharmacokinetics

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ABSTRACT

Among all the antimalarial agents, artemisinin and its semi synthetic family of analogs are the most potent antimalarials available for the treatment of *Plasmodium falciparum* infections. But these analogs have a few issues such as shorter half-lives and low oral bioavailability values. In order to overcome these inherent problems, novel artemisinin analogs were synthesized from 7β-hydroxy artemisinin by the Department of Medicinal Chemistry, University of Mississippi using a new synthesis mechanism. Out of all the 7β-hydroxy artemisinin analogs synthesized, 7β-hydroxy artemisinin carbamate (ARB-89) was chosen as a lead compound because of its high *in vitro* and *in vivo* activity. In this manuscript, a sensitive and rapid ultra-performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) method was developed and validated for the quantification of ARB-89 in rat serum. The analysis was carried out on an Acquity™ UPLC BEH C_{18} column (1.7 μm, 2.1 mm × 50 mm) with a flow rate of 0.3 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer in positive electrospray ionization (ESI) mode. The selected mass-to-charge (m/z) ratio transitions used in the multiple reaction monitoring (MRM) for ARB-89 and artemisinin (internal standard) were m/z 778.4 > 253.4 and m/z 283.4 > 151.1 respectively. The calibration curve was linear from 1.00 ng/mL to 10.0 μg/mL (r^2 = 0.999). A simple protein precipitation method was used for extraction. Moreover, the inter-day and intra-day precision values were found to be less than 15%. The recoveries of the method ranged from 94.0% to 96.7% at three concentrations. ARB-89 in rat serum was found to be stable at room temperature for 12 h. This method was successfully used to quantitate the novel antimalarial compound ARB-89 after intravenous and oral administration to rats.

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1. Introduction

Malaria continues to be a major health problem in many areas of the world and was reported to cause about 300 million illnesses and at least 1 million deaths a year [1,2]. Around 109 countries were endemic for malaria in 2008, 45 within the WHO African region [3,4]. This disease affects not only individuals but also the entire nation by reducing productivity and results in an estimated 1.3% average loss of economic growth annually [3]. The widespread resistance to commonly used drugs (chloroquine, quinine, mefloquine, primaquine) [5] has focused attention on the artemisinin group of compounds. Artemisinin and its derivatives such as artesunate and arteether are found to be rapidly acting and well tolerated endoperoxide compounds [6]. Artemisinin has the ability to quickly lower parasite levels even in severe cases of cerebral malaria. Additionally, artemisinin has demonstrated activity against drug resistant strains of *Plasmodium falciparum* [7]. However, there are still some issues associated with artemisinin compounds including a short half-life, limited bioavailability, and poor water solubility [8,9]. These inherent problems prompted us to synthesize novel antimalarial artemisinin derivatives with better pharmacokinetic profiles.

As a part of our ongoing investigation to develop more potent antimalarial agents based on artemisinin, we have designed and synthesized many novel 7β-hydroxy artemisinin derivatives. The

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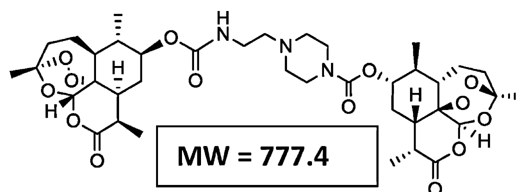

Fig. 1. Chemical structure of artemisinin analog ARB-89.

microbial fermentation of artemisinin by the fungus *Cunninghamella elegans* has resulted in the production of hydroxylated analog, 7β-hydroxy artemisinin [10] and that was used as a starting material for synthesizing novel artemisinin analogs. Since amino functionality is known to increase bioavailability of drugs, various amino derivatives were prepared from 7β-hydroxy artemisinin and out of all the derivatives, ARB-89 (7β-hydroxy artemisinin carbamate (Fig. 1)) has shown increased antimalarial activity.

In the case of ARB-89, 7β-hydroxy artemisinin was first treated with triphosgene in the presence of 4-dimethylaminopyridine to give the chloroformate intermediate that was subsequently treated with 2-(1-piperazinyl) ethanamine to produce the target compound ARB-89 (unpublished results [11]).

Among all the analogs, ARB-89 has exhibited excellent *in vitro* antimalarial activity relative to artemisinin and its existing derivatives in *P. falciparum* and the *W2* clone. *In vivo* activity of ARB-89 was also tested against *P. berghei N* in 20 g male CD1 mice. For example, ARB-89 has shown >96% suppression in parasitemia at a dose of 3.3 mg/kg as compared to 94% suppression shown by artesunate at the same dose level with no signs of cytotoxicity observed (unpublished results [11]). Based on the superior biological activity, this compound was selected for further development as a potential antimalarial lead. During the development of a new drug candidate, it is essential to obtain early information regarding its pharmacokinetic parameters as early as possible [12,13]. To further understand and explore the pharmacokinetic profile and therapeutic effect of the ARB-89, a sensitive and high throughput method is required for routine analysis in biological samples.

The aim of this study was to develop and validate a UPLC/MS/MS method to measure clinically relevant concentrations of ARB-89 in serum after intravenous and oral administration of the compound to the rats. Due to the fact that artemisinin and its analogues have no appropriate chromophores [14,15], they sometimes require lengthy derivatization techniques for quantitation. These derivatizing conditions might not be stable for all the artemisinin analogs [14] and can cause the parent compound to decompose. The UPLC/MS/MS was chosen as the analytical method for ARB-89 because it was known to be one of the powerful separation and detection techniques in the bioanalytical field [16]. Using this method, a high sample throughput was achieved by a simple sample preparation and short run times. This method was found to be very helpful in assessing the basic pharmacokinetic behavior of ARB-89 and could be further used to investigate and explore the therapeutic potential of the compound in the future.

2. Experimental

2.1. Reagents and chemicals

Artemisinin (99.7% purity) was obtained from Sigma–Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol, chloroform, tertiary butyl methyl ether (TBME) and HPLC grade water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Reagent grade formic acid (95% purity) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Drug-free rat serum was bought from Innovative Research Inc. (Southfield, MI, USA). Precanulated Male Sprague-Dawley rats (200–220 g) were obtained from Harlan Company (Indianapolis, IN, USA). ARB-89 was synthesized by Dr. Mitchell Avery's group from the Department of Medicinal Chemistry at University of Mississippi. The purity of the compound was proved to be greater than 98% by NMR and HPLC.

2.2. Apparatus and operating conditions

2.2.1. Liquid chromatography

The chromatographic separations were carried out on Acquity™ UPLC system (Waters, Corp., Milford, MA, USA). It was equipped with a binary solvent manager, vacuum degasser, thermostatted column compartment and an auto sampler. Chromatographic separations were performed on a Waters Acquity™ UPLC BEH C_{18} column (1.7 μm, 2.1 mm × 50 mm). The mobile phase consisted of acetonitrile and 0.3% formic acid (60:40, v/v). The flow rate was 0.3 mL/min, the sample injection volume was 10 μL and the duration of the run was 3 min. The column temperature was maintained at 25 °C.

2.2.2. Mass spectrometry

The mass spectrometric detection was carried out on a Micromass Quattro Micro™ API triple-quadrupole system (Waters Corp., Manchester, UK). The MS system was controlled by version 4.0 MassLynx software. Ionization was performed in the positive electrospray mode. The optimal MS/MS conditions were the following: capillary voltage 3.9 kV; cone voltage 40 V; extractor voltage 2 V; RF lens voltage 0.2 V. The source and desolvation temperatures were maintained at 100 °C and 350 °C, and the desolvation and cone gas flows were held at 650 and 35 L/h.

The selected mass-to-charge (*m*/*z*) ratio transitions of ARB-89 and internal standard $[M+H]^+$ used in the Multiple reaction monitoring (MRM) were *m*/*z* 778.4 > 253.4 and *m*/*z* 283.4 > 151.1 respectively. The collision energy for fragmentation of the precursor ions was set at 28 and 22 eV for ARB-89 and IS, respectively. The total dwell time was set at 500 ms.

2.3. Preparation of standards and quality control samples

Individual standard stock solutions of the ARB-89 (1 mg/mL) and the internal standard (0.2 mg/mL), were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving and diluting with acetonitrile. A calibration standard for ARB-89 was prepared by spiking 5 mL of blank rat serum with 100 μL of ARB-89 stock solution. This provided a serum standard with a concentration of 20 μg/mL of the ARB-89. Further dilutions were made from this stock solution with blank serum to afford serum standards in the range of 1.00 ng/mL to 10.0 μg/mL. The final concentration of internal standard in the serum samples was 100 ng/mL. On each analysis day a calibration curve was constructed using freshly prepared calibration standards.

The QC samples were used to assess the accuracy and precision of the assay method. The quality control (QC) samples were prepared using the same process as the calibration samples. Four concentrations (1, 3, 500 ng/mL and 7.5 μg/mL, representing lower limit of quantification, low QC, mid QC and high QC) were prepared. All the calibration and QC plasma samples were then extracted by the method described in Section 2.4. The QC samples were stored along with the test samples at −80 °C until needed.

2.4. Serum sample preparation

Serum samples, including calibration standards and QC samples, were extracted using acetonitrile. An aliquot of 90 μL of rat serum and a 10 μL of the internal standard solution were placed in an eppendorf micro tube for processing. A mixture of 200 μL of

acetonitrile and methanol (7:3, v/v) was added to precipitate the serum proteins and the micro centrifuge tube was vortex mixed for 10 min and centrifuged for 20 min at 10,000 rpm. The supernatant was collected, filtered through a 0.45 μm syringe filter and analyzed using UPLC/MS/MS.

2.5. Method validation

The method validation assays were performed as per the US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance [17]. The validation of the UPLC/MS/MS method included selectivity, recovery, matrix effect, linearity, lower limit of quantification (LLOQ), precision, accuracy and stability studies.

2.5.1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Selectivity was evaluated by comparing the chromatograms of six different batches of the blank serum with the corresponding standard serum samples spiked with ARB-89 and the internal standard, as well as the rat serum samples after intravenous administration of ARB-89.

2.5.2. Linearity and lower limit of quantification (LLOQ)

A calibration curve was constructed from serum standards at eight concentrations of ARB-89 ranging from 1.00 ng/mL to 10.0 μg/mL. A calibration curve was constructed by plotting the peak area ratio of ARB-89/IS *versus* nominal concentration of ARB-89. The correlation coefficient and linear regression equation were used for the determination of analyte concentration in the samples. A weighted ($1/x^2$) linear least-squares regression was used as the mathematical model. The calibration was processed with the MassLynx 4.0 QuanLynx software. The accuracy for calibration standards should be within ±15% of their respective nominal value at each concentration except for the LLOQ (lower limit of quantification), where it should be within ±20% of the nominal value. The LLOQ was determined as the lowest concentration that produced an S/N of 5. The limit of detection (LOD) was determined as the lowest concentration that produced an S/N of 3.

2.5.3. Accuracy and precision

Intra-day accuracy and precision were evaluated by analyzing the four QC concentrations (1, 3, 500 ng/mL and 7.5 μg/mL) with five determinations per concentration on the same day. The inter-day accuracy and precision were evaluated by the analysis of the four QC concentrations (1, 3, 500 ng/mL and 7.5 μg/mL) with five determinations per each concentration over 3 days. Precision was based on the criteria that the RSD for each concentration should be not more than 15%, except for LLOQ (not to be more than 20%). For accuracy, the mean value should not deviate by more than ±20% of the actual concentration.

2.5.4. Extraction recovery and matrix effect

The mean extraction recovery of ARB-89 was measured at three different concentration levels (3, 500 ng/mL and 7.5 μg/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.

The elution of endogenous components may affect the ionization efficiency of the analyte. The matrix effect was determined by comparing the peak areas of ARB-89 (3, 500 ng/mL and 7.5 μg/mL) extracted from six different batches of the serum (A) and the corresponding standard solutions evaporated directly and reconstituted in the mobile phase (B). The ratio ($A/B \times 100$)% was calculated to evaluate the matrix effect and the matrix effect of internal standard was also evaluated using the same procedure at 100 ng/mL.

2.5.5. Stability

2.5.5.1. Freeze–thaw stability. Freeze–thaw stability of the serum samples containing ARB-89 was evaluated by subjecting the QC samples to three freeze–thaw cycles. The QC samples ($n = 6$) at three final concentrations (3, 500 ng/mL and 7.5 μg/mL) were stored at −20 °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 samples were processed and compared with the freshly prepared QC samples and the accuracy values were calculated after each cycle.

2.5.5.2. Short term stability. Short-term temperature stability of the serum samples was evaluated by storing the QC samples ($n = 6$) at room temperature (25 °C) for 12 h. At 0, 4, 6 and 12 h, the samples were processed, analyzed, compared with the freshly prepared QC samples and then the accuracy values were calculated.

2.5.5.3. Post preparative stability. Post preparative stability of the serum samples was determined by extracting the QC samples ($n = 6$) at three concentrations (3, 500 ng/mL and 7.5 μg/mL) as per the developed method and incubated in auto sampler vials at 4 °C for up to 3 days. All these QC samples were processed and compared with the nominal values and the accuracy values were calculated.

2.5.5.4. Stability in rat serum. Stability of the ARB-89 in rat serum was also carried out at three different concentrations (3, 500 ng/mL and 7.5 μg/mL) in triplicate. Stability of carbamate was assessed after extracting with acetonitrile and methanol (7:3, v/v) over a 24 h period. Stability of ARB-89 in rat serum alone was also determined after storage at room temperature and 4 °C for 5 days. For this stability evaluation, the concentration on day 1 was compared with that of day 5 and the percentage was calculated using the equation ((day 2 or 5 concentration/day 1 concentration) × 100).

2.6. Formulation

The intravenous formulation of ARB-89 was prepared in 1 mL of 10% Tween-80, 10% ethanol and 80% saline. The intravenous formulation was administered to rats ($n = 6$) at a dose of 5 mg/kg. The oral formulation of ARB-89 was prepared in 1 mL of 10% Tween-80, 10% peanut oil, 20% ethanol and 60% saline. The oral formulation was administered to rats ($n = 6$) at a dose of 100 mg/kg.

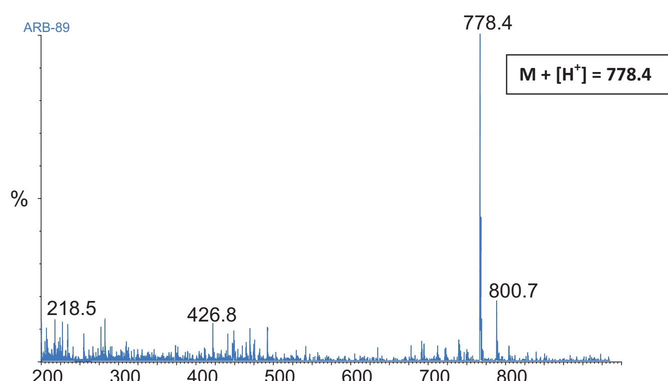

Fig. 2. Molecular mass spectrum of ARB-89.

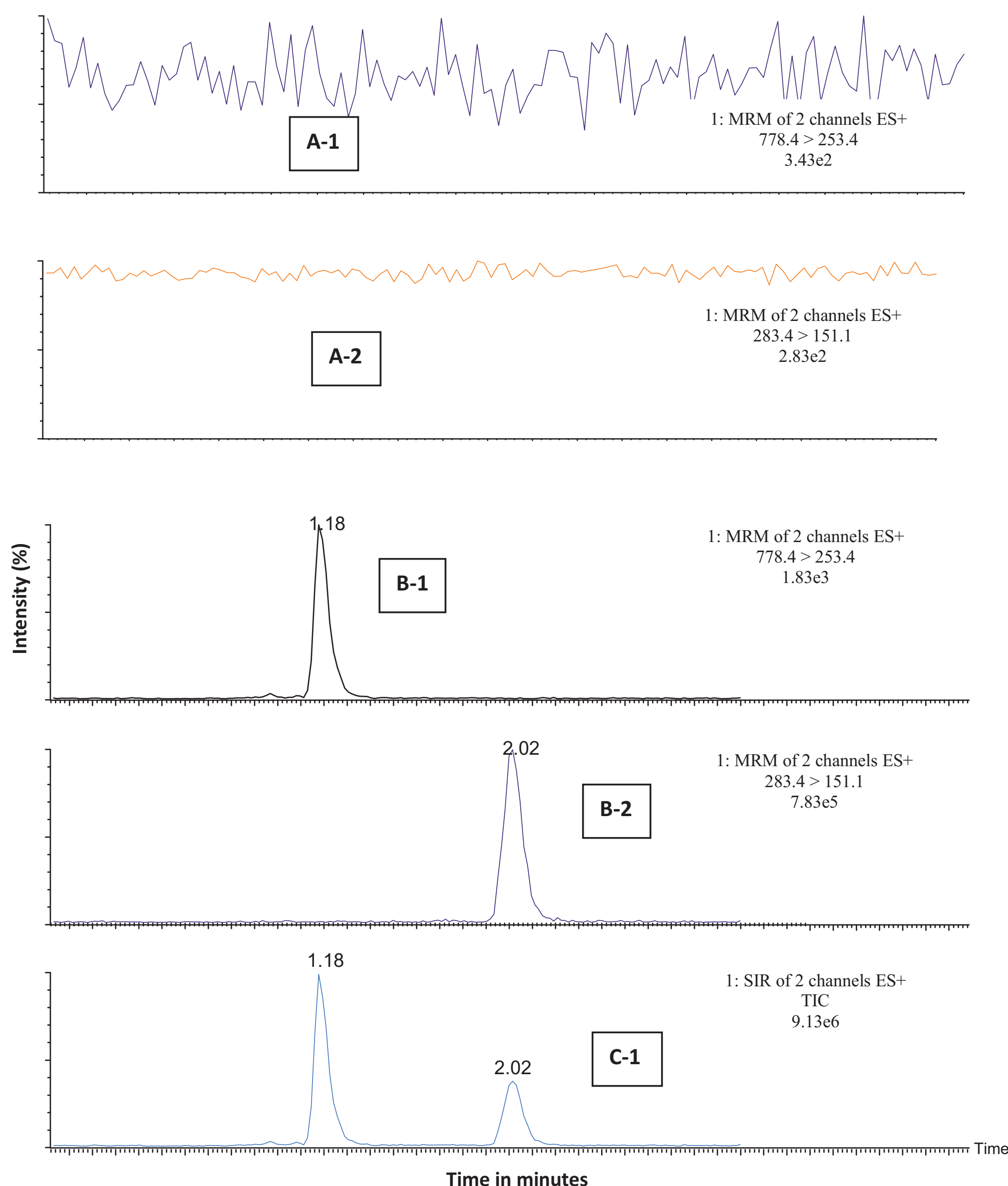

Fig. 3. Typical chromatograms of (A-1) and (A-2) Blank serum. (B-1) Blank serum spiked with ARB-89 at 1 ng/mL (LLOQ). (B-2) Blank serum spiked with IS (100 ng/mL). (C-1) Serum sample collected at 30 min after intravenous administration of ARB-89 spiked with IS (artemisinin).

2.7. Animal studies

The assay method described above was applied to study the pharmacokinetics of ARB-89 in rat serum after intravenous administration. All the experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi. Precanulated Sprague-Dawley rats (200–220 g) were obtained from Harlan Company (Indianapolis, IN). These rats were housed in standard metabolism cages and the rats were fasted for 12 h before dosing but allowed free movement and access to water during the whole experiment. The i.v. formulations were administered *via* the jugular vein cannula and the oral (p.o.) formulations were administrated using gavage, after which the cannula was flushed with 0.2 mL heparinized saline to ensure complete administration of the dose. First group of rats ($n = 6$) were dosed intravenously at a dosage of 5 mg/kg. Second group of rats ($n = 6$) were dosed orally at a dosage of 100 mg/kg. Blood samples were collected using the indwelling cannula before dosing and at predetermined time intervals after dosing. Serum was separated from the blood samples by centrifugation at 3000 × *g* for 10 min at 4 °C and stored at −80 °C until analysis using UPLC/MS/MS.

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 $AUC_{0\to t}$ values (t being the time of the last plasma concentration measured) were calculated by the linear trapezoidal rule. The first order rate constant, λ_z, 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. Results and discussion

3.1. Ultra-performance liquid chromatography and mass spectrometry

High-performance liquid chromatography combined with UV, MS and electro chemical detectors has been widely used for the

Table 1
Intra and inter day precision and accuracy of ARB-89 ($n=5$) in rat serum.

Spiked concentration (ng/mL)	Intra-day precision and accuracy ($n=5$)		Inter-day precision and accuracy ($n=5$)	
	Accuracy (%) ± SD	RSD (%)	Accuracy (%) ± SD	RSD (%)
1	94.3 ± 2.1	2.2	94.0 ± 1.2	1.3
3	97.4 ± 2.5	2.6	95.7 ± 1.3	1.4
500	97.5 ± 1.1	1.1	97.2 ± 2.6	2.7
7500	99.3 ± 0.2	0.2	99.1 ± 0.1	0.1

RSD, relative standard deviation; accuracy = (measured concentration/nominal concentration) × 100.

analysis of artemisinin analogues in biological samples [18,19]. However, they were not fast and sensitive enough to quantitate the large number of samples in biological matrices, necessary to accomplish in a pharmacokinetic study in small animals [19,20]. An improvement in chromatographic performance has been achieved by the introduction of ultra-performance liquid chromatography (UPLC). Thus, UPLC takes full advantage of chromatographic principles to run separations using columns packed with smaller particles (1.7 μm), with superior resolution and a shorter analysis time [21]. A 50 mm column subjected to an isocratic flow rate of 0.3 mL/min for 3 min was used for the chromatographic separation. The mobile phase optimization was accomplished by comparing various solvent systems composed of mixtures of methanol or acetonitrile with formic acid, acetic acid, ammonium acetate and ammonium hydroxide solutions. The mobile phase consisting of a mixture of acetonitrile: 0.1% formic acid was found to be suitable for separation and ionization of ARB-89 and internal standard. The presence of formic acid was found to increase the ionization of both the compounds.

The optimization of the MS parameters was carried out by infusing a standard solution *via* a syringe pump into the mobile phase in the electrospray positive ion mode. The corresponding molecular spectrum of ARB-89 was shown in Fig. 2.

Very narrow chromatographic peaks were generated by UPLC with peak widths less than 5 s and this has resulted in an increase in the chromatographic efficiency, there by faster separations and increased sensitivity. Both the compounds were rapidly eluted with in a run time of 3 min (Fig. 3).

3.2. Selection of extraction method

Protein precipitation technique was used for extracting the drug from serum. Several solvents (acetonitrile, methanol, chloroform and methyl t-butyl ether) were tested for the extraction. Recovery using methanol (48%), methyl t-butyl ether (67%) and chloroform (82%) were found to be less than acetonitrile (95%). Therefore, acetonitrile was selected as the extraction solvent in serum due to its high recovery.

3.3. Optimization of the intravenous and oral formulations

The artemisinin analog ARB-89 is a highly non-polar compound with calculated XLogP of 3.51 (ToxPredict by Toxtree, Version of 05 August 2011). Our aim was to find a formulation that would increase the solubility of ARB-89 and decrease its precipitation in blood serum. In many cases, it was proved that the use of co-solvents to prepare solution based formulations of nonpolar drugs is a simple and potentially effective way to achieve high concentration of drugs [22]. During our experiments, several co-solvents such as PEG 400, PEG 300, propylene glycol, glycerin, ethanol, Cremophor EL, DMSO, Tween 80 and saline were tested as vehicles to increase the solubility of ARB-89. Among them glycerin and propylene glycol were reported to produce significant hemolysis and muscle damage [18]. Cremophor EL was too viscous to be used for intravenous formulation. PEG 400 and PEG 300 were not able to solubilize ARB-89 completely. Finally, the composition of 10% Tween-80, 10% ethanol and 80% saline was chosen to formulate ARB-89 for the intravenous administration. The oral formulation of the ARB-89 was prepared in 1 mL of 10% Tween-80, 10% peanut oil, 20% ethanol and 60% saline and both the formulations were proved to be safe.

Table 2
Recovery of ARB-89 ($n=3$) from rat serum.

Spiked concentration (ng/mL)	Recovery (%) ($n=3$)	
	Mean ± SD	RSD
3	96.7 ± 1.2	1.2
500	94.0 ± 1.1	1.1
7500	96.1 ± 2.4	2.5

RSD, relative standard deviation; recovery = response of standard spiked before extraction/response of standard spiked after extraction.

3.4. Method validation

3.4.1. Selectivity

Analysis of the extracted serum samples showed no interference from endogenous substances at the retention times of either ARB-89 or the internal standard. There was a good base line separation of ARB-89 and the internal standard extracted from the rat serum. Representative chromatograms of blank serum, blank serum spiked with ARB-89 and internal standard and extracted serum samples are shown in Fig. 3.

3.4.2. Linearity and LLOQ

Calibration curves were constructed from serum standards at eight concentrations of ARB-89 ranging from 1.00 ng/mL to 10.0 μg/mL. The ratio of peak areas of ARB-89 to that of the IS was used for quantification. The calibration model was selected based on the analysis of the data by linear regression with intercepts and $1/x^2$ weighting factor. The calibration curve was linear over a concentration range from 1.00 ng/mL to 10.0 μg/mL with a correlation coefficient of 0.999, indicating good linearity. A typical calibration

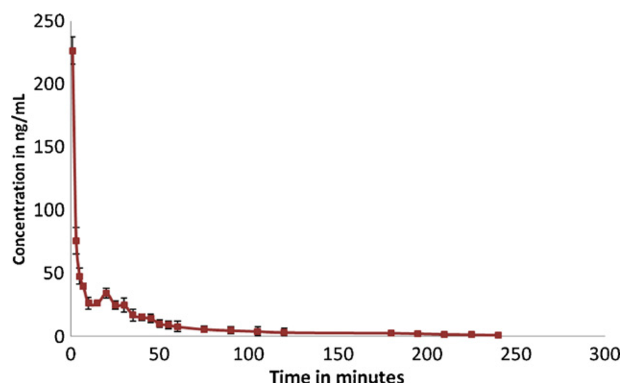

Fig. 4. Mean serum concentration time profile after intravenous administration of ARB-89 to rats ($n=6$) at 5 mg/kg.

Table 3
Stability studies of ARB-89 ($n=6$) in rat serum.

	Time (h)	Concentration (ng/mL) ($n=6$)					
		3		500		7500	
		Accuracy (%) ± SD	RSD (%)	Accuracy (%) ± SD	RSD (%)	Accuracy (%) ± SD	RSD (%)
Short-term	0	98.3 ± 1.7	1.7	97.2 ± 0.7	0.7	98.3 ± 1.3	1.3
	4	97.1 ± 2.2	2.3	96.8 ± 2.3	2.4	97.8 ± 2.3	2.3
	6	96.3 ± 2.4	2.5	96.5 ± 1.7	1.8	93.4 ± 2.5	2.7
	12	96.5 ± 1.7	1.8	96.0 ± 2.2	2.3	95.2 ± 3.4	3.6
	Cycle (s)	Concentration (ng/mL) ($n=6$)					
		3		500		7500	
		Accuracy (%) ± SD	RSD (%)	Accuracy (%) ± SD	RSD (%)	Accuracy (%) ± SD	RSD (%)
Freeze–thaw	0	97.9 ± 2.0	2.0	97.2 ± 3.1	3.2	98.4 ± 2.3	2.3
	1	98.5 ± 3.1	3.1	94.3 ± 4.2	4.4	98.2 ± 2.1	2.1
	2	94.3 ± 2.3	2.4	96.3 ± 3.0	3.1	96.0 ± 4.0	4.2
	3	95.4 ± 3.4	3.6	94.5 ± 3.2	3.4	92.3 ± 3.4	3.7
	Time (h)	Concentration (ng/mL) ($n=6$)					
		3		500		7500	
		Accuracy (%) ± SD	RSD (%)	Accuracy (%) ± SD	RSD (%)	Accuracy (%) ± SD	RSD (%)
Post preparative	0	98.2 ± 2.2	2.2	96.2 ± 3.2	3.3	97.4 ± 2.5	2.6
	24	98.1 ± 3.5	3.6	98.3 ± 2.3	2.3	98.4 ± 3.1	3.1
	48	97.8 ± 2.4	2.4	96.1 ± 3.1	3.2	96.5 ± 2.8	2.9
	72	94.2 ± 2.3	2.4	97.1 ± 2.8	2.9	96.2 ± 1.4	1.4

RSD, relative standard deviation; accuracy = (measured concentration/nominal concentration) × 100.

equation for the calibration curve obtained from 1.00 ng/mL to 10.0 μg/mL was $y = 0.00184x - 0.00057$. The LLOQ was found to be 1 ng/mL, with an accuracy of 97.0%. The LOD was found to be 0.2 ng/mL.

3.4.3. Accuracy and precision

The results of the intra-day and inter-day accuracy and precision are listed in Table 1. The intra-day precision for LLOQ, low, mid and high concentrations was 2.2%, 2.6%, 1.1% and 0.2%, respectively, and the accuracy ranged from 94.3% to 99.3%. The inter day precision for LLOQ, low, mid and high concentrations was 1.3%, 1.4%, 2.7% and 0.1%, respectively and the accuracy ranged from 94.0% to 99.1%. The precision and accuracy of the method met the FDA criterion for the analysis where the RSD values were below 15% at all the concentration levels and below 20% for LLOQ. The accuracy values were also within the range of 80–120% at all the concentration levels.

3.4.4. Recovery and matrix effect

The mean recoveries of artemisinin based carbamate ranged from 94.0% to 96.7% at three QC levels and the extraction recovery of IS was 92.1 ± 2.1% at 100 ng/mL. Recovery values were listed in Table 2.

In the case of the matrix effect ($n=6$), the ratios obtained were 91.2 ± 8.1%, 92.4 ± 2.6% and 92.9 ± 2.4% at the concentrations of 3, 500 ng/mL and 7.5 μg/mL respectively. The same effect was evaluated for the IS and the ratio was 92.4 ± 2.3% at 100 ng/mL. The results indicated that all the ratios ($A/B \times 100$)% defined as in Section 2 were in between 85 and 115% which means that there is no matrix effect from endogenous serum components on the ionization of the ARB-89 and the IS.

3.4.5. Stability

Initially, stability of carbamate was established in rat serum. Carbamate was stable in serum, after sample preparation with acetonitrile and methanol (7:3, v/v) at room temperature over a 24 h period. The ARB-89 was stable in serum alone for at least 5 days at both room temperature and 4 °C. The concentration of ARB-89 was 90.3% and 94.6% of the original concentration after day 5 stored at room temperature and 4 °C.

The results of freeze–thaw and short-term storage stability are listed in Table 3. The ARB-89 was found to be stable after three freeze–thaw circles at three different concentrations. ARB-89 was found to be stable after storing at room temperature for 12 h. The mean accuracies from the nominal concentrations were between 93% and 98% at three different concentrations. Extracts of the QC samples were found to be stable in the auto-sampler for at least 72 h. The results from all the stability studies indicate that ARB-89 is stable at all stages of determination.

3.5. Application to pharmacokinetic study

The present method was successfully validated and applied to quantitate ARB-89 in the serum samples after intravenous and oral administration to Sprague Dawley rats. Fig. 4 shows the intravenous plasma concentration time profile for ARB-89 in rats. Fig. 5 shows the oral plasma concentration time profile for ARB-89 in rats.

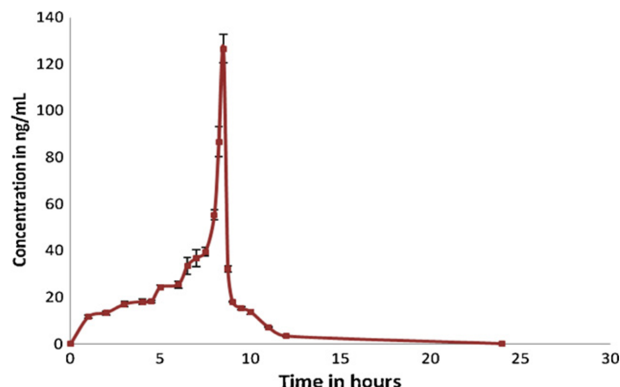

Fig. 5. Mean serum concentration time profile after oral administration of ARB-89 to rats ($n=6$) at 100 mg/kg.

Table 4
Pharmacokinetic parameters of ARB-89 after intravenous administration of 5 mg/kg to Sprague Dawley rats ($n = 6$) following non compartmental model using WinNonlin software.

Parameters	Unit	Mean	%CV
C_{max}	ng/mL	226	4.8
$t_{1/2}$	min	45	11.1
V_d	L/kg	152	21.6
CL_p	L/h/kg	137	14.9
$AUC_{0\to t}$	min ng/mL	3225	5.0

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; %CV, coefficient of variation.

Table 5
Pharmacokinetic parameters of ARB-89 after oral administration of 100 mg/kg to Sprague Dawley rats ($n = 6$) following non compartmental model using WinNonlin software.

Parameters	Unit	Mean	% CV
C_{max}	ng/mL	126	12.8
$t_{1/2}$	min	53	9.8
T_{max}	h	8.5	0.0
V_d	L/kg	430	44.2
CL_p	L/h/kg	361	24.2
$AUC_{0\to t}$	min ng/mL	15,105	35.2

C_{max}, peak plasma concentration; $t_{1/2}$, terminal elimination half life; T_{max}, time of maximum peak plasma concentration; AUC, area under the plasma concentration–time curve; V_d, apparent volume of distribution; CL_p, plasma clearance; %CV, coefficient of variation.

The corresponding results of the pharmacokinetic parameters are shown in Tables 4 and 5. After intravenous and oral administration of the compound, close and continuous visual monitoring of the animals revealed that there were no severe acute toxicity responses as none of the animals showed any signs of behavioral or neurological toxicity during the entire study period. The PK data analysis showed that ARB-89 has a fast clearance rate and large volume of distribution. This might suggest that ARB-89 quickly leaves the central compartment and is distributed to the tissues. Furthermore, the short half-life ($t_{1/2}$) of ARB-89 compared to artemisinin [21] also indicates that the compound is removed rapidly from the blood. Oral bioavailability of ARB-89 was found to be 23.4%. Oral bioavailability of ARB-89 was found to be better than artemisinin and its marketed analogs such as artemisinin, artenimol and artemether (ranging from 19% to 22%) [23], Even though half-life of the compound was not improved, this kind of compounds (combining two artemisinin compounds with a bridge) has future potential due to their high activity. Taking this compound as lead, novel analogs can be further synthesized to improve the pharmacokinetic properties. Method development and evaluation of pharmacokinetic properties of this compound will help in leading a path for synthesizing novel analogs with improved pharmacokinetic profile.

4. Conclusion

In conclusion, a rapid and sensitive UPLC/MS/MS method was developed, validated and successfully applied to evaluate the pharmacokinetic parameters after intravenous and oral administration of ARB-89 to rats. The sample preparation procedure involved a simple protein precipitation method and the analysis required only 90 μL of serum and a short run time of 3 min, which is very advantageous in a pharmacokinetic study. This method provided a number of analytical advantages such as excellent sensitivity, linearity, precision and accuracy. Therefore this UPLC/MS/MS method can be considered as an excellent technique to further evaluate the pharmacokinetic properties and there by the therapeutic potential of the novel antimalarial compound ARB-89.

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