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Journal of Chromatography B, 783 (2003) 391–399
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatographic–mass spectrometric method for the determination of α-,β-arteether in rat serum☆

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Received 22 May 2002; received in revised form 20 August 2002; accepted 9 September 2002

Abstract

This study reports the development and validation of a sensitive and selective assay method for the determination of α-,β-arteether in rat serum by liquid chromatography–mass spectrometry. The mobile phase was composed of methanol–0.1 m*M* sodium acetate (pH 5) (80:20%) at a flow-rate of 1 ml min^{-1} and chromatographic separations were achieved on a Ultracarb, 5 ODS 20, Phenomenex column (5 μm, 30 mm×4.6 mm I.D.). The total effluent from the column was split so that one-tenth was injected into the electrospray LC–MS interface. ESI-MS analysis was carried out using a Micromass Quattro II Triple Quadrupole Mass Spectrometer equipped with an electrospray source. The MS analysis was carried out at a cone voltage of 52 V with a scan range of 100–400 Da. The analytes were quantified from the $[M+Na]^+$ ion chromatograms of α-,β-arteether at *m*/*z* 335 and artemisinin at *m*/*z* 305. A simple liquid–liquid extraction with 2×2 ml *n*-hexane was used to isolate α-,β-arteether from rat serum. The method was validated in terms of recovery, linearity, accuracy and precision (within- and between-assay variation). The recovery from spiked control samples ranged from 88.41 to 96.17% with a maximum CV of 10.8% for α-arteether and 69.83–79.69% with a maximum CV of 17.06% for β-arteether. Linearity in serum was observed over the range 20–320 ng ml^{-1}. Percent bias (accuracy) was well within the acceptable range. Within- and between-assay precision were less than 15%. The assay method described here is being applied to study the pharmacokinetics of CDRI developed intramuscular formulation Emal (α-/β-arteether in the ratio of 30:70) in rats. The method is sensitive enough to monitor α-,β-arteether up to 24 h after a single 30 mg kg^{-1} i.m. dose.

Keywords: α-,β-Arteether

1. Introduction

Malaria is by far the world's most important tropical parasitic disease, and kills more people than any other communicable disease except for tuberculosis. According to WHO Fact Sheet No. 94, malaria is a public health problem in more than 90 countries, inhabited by a total of some 200–400 million people accounting for 40% of world's population. World-wide prevalence of the disease is estimated to be in the order of 300–500 million clinical cases each year. Isolated in 1972 from the Chinese medicinal plant *Artemisia annua* Linn., artemisinin (Fig. 1c) is a novel antimalarial with a sesquiterpene lactone structure containing an internal

☆CDRI Communication No. 6281

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00701-8

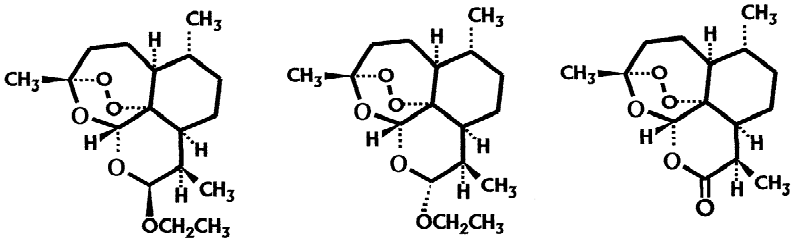

Fig. 1. Structure of (a) β-arteether; (b) α-arteether; (c) artemisinin (I.S.).

endoperoxide linkage, which is essential for the activity [1]. β-Arteether (Fig. 1a), the ethyl ether derivative of the natural product Artemisinin is under investigation by the World Health Organization (WHO) as a new antimalarial drug because of its high level of blood schizonticidal activity against chloroquine-resistant *Plasmodium falciparum* malaria [2] and especially cerebral malaria [3].

During a 1992 World Health Organization conference, it was emphasized that efforts should be made to develop robust analytical methods for monitoring pharmacologic and idiosyncratic toxicity in patients treated with these analogues [4]. The development of a selective analytical method for the determination of arteether in biological fluids poses challenging problems. It is thermally labile, lacks ultraviolet (UV) absorption or fluorescent chromophores, and does not possess functional groups with potential for derivatization [5]. Several extraction procedures have been published in the literature for the determination of these types of compounds in biological fluids. Usually two different approaches have been used for the determination of these types of compounds in biological fluids. The first approach is pre-column acid- or base-catalyzed decomposition to UV-absorbing compounds followed by the HPLC of the decomposition products [6–8] and the second, HPLC with reductive electrochemical detection (LC-EC) using thin-layer gold-mercury amalgam or dropping mercury electrode or glassy carbon electrode [9–11]. The first approach lacks specificity in that the metabolites of the drug are also converted to identical UV-absorbing products in many instances. Liquid chromatography (LC) coupled with electrochemical detection (EC) is tedious and cannot be applied in routine analysis. Inherent difficulties of the method are that it requires rigorous deoxygenation of the samples and mobile phase. However, the need for special laboratory facilities and highly trained and experienced technicians makes the assay in reduction mode available in only a limited number of institutes world-wide [5]. Supercritical fluid chromatography coupled with electron-capture (ECD) and flame ionization (FID) detectors as well as evaporative light scattering detector (ELSD) were also developed [12,13]. Finally, radioimmunoassay (RIA) techniques were used but showed a lack of selectivity [14].

Liquid chromatography coupled to mass spectrometry (LC–MS) is known to be a powerful separation and detection technique in a large number of analytical fields, and particularly for the detection of drugs in biological fluids [15–19]. High sensitivity and selectivity of mass spectrometry present several advantages in comparison with either LC/EC or LC–UV [20]. Earlier literature reports the quantification of the glucuronides of the hydroxy metabolites of arteether in rat plasma and urine by HPLC–MS. Structural elucidation of the microbial and mammalian metabolite of arteether has also been reported using thermospray LC–MS [21]. To, our knowledge, LC–MS has never been used to quantify arteether in plasma or serum for the purpose of pharmacokinetic characterization. The development of the atmospheric pressure ionization (API) source is an important breakthrough that has enabled coupling LC with MS conveniently. API when operated in the electrospray ionization (ESI) format is unique in that it has a great potential for the analysis of a variety of both small and large molecules at femtomole sensitivities. The utility of HPLC–ESI-MS

has been demonstrated for a wide range of applications in bioanalytical, pharmaceutical, and environmental fields [22].

This paper presents for the first time the development and validation of an assay method for the simultaneous estimation of α-,β-arteether in rat serum by liquid chromatography–mass spectrometry (LC–MS), using an electrospray interface operated in positive ion mode. In this routine method, a high sample throughput is achieved by simple sample preparation and by short chromatographic run times in the isocratic mode. This analytical method was applied to estimate the levels of α-,β-arteether in rats following an intramuscular dose of Emal 30 mg kg^{-1}.

2. Experimental

2.1. Chemicals and materials

Pure reference standards of α-arteether and β-arteether, Emal (150 mg in 2 ml) were obtained from Themis Chemicals Limited, Mumbai, India. Artemisinin was obtained from the Medicinal Chemistry Division, Central Drug Research Institute (CDRI), Lucknow, India. Hexane and methanol, HPLC grade, UltimAR™, was obtained from Mallinckrodt (Kentucky, USA). Hexane was further purified by slow distillation before use. Sodium acetate was obtained from JT Baker (Phillipsburg, NJ, USA). Purified water of 18.2 MΩ cm was obtained from the Milli-QPLUS PF system. Drug-free rat serum was obtained from young, healthy male Sprague–Dawley rats procured from Laboratory Animal Services Division of CDRI.

2.2. Liquid chromatography

A Jasco PU980, intelligent pump was used to deliver a premixed mobile phase composed of methanol–0.1 m*M* sodium acetate (80:20% v/v) at a flow-rate of 1 ml min^{-1}. The mobile phase was degassed in an ultrasonic bath (Bransonic Cleaning Co., USA) prior to the analysis. Chromatographic separations were achieved on a Ultracarb, 5 ODS 20, Phenomenex column (2320W.205th Street, Torrance, CA, USA) of 30 mm×4.6 mm I.D., 5-μm particle size. The samples were injected through a manual injector (Rheodyne model No. 7125, Cotati, USA) fitted with a 20-μl loop. Automated data acquisition was triggered using contact closure signals of the manual injector. The total effluent from the column was split so that one-tenth was injected into the electrospray LC–MS interface.

2.3. Mass spectrometry analysis

ESI-MS analysis was carried out using a Micromass Quattro II Triple Quadrupole Mass Spectrometer equipped with an electrospray source. Data acquisition and analyses were carried out using MassLynx version 3.1 software. Nitrogen was used as both the nebulizing gas (10 l h^{-1}) and as curtain gas (250 l h^{-1}). The ESI capillary was set at 3.5 kV while the MS analysis was carried out at a cone voltage of 52 V with a scan time of 1 s and inter-scan delay of 0.1 s and a scan range of 200–500 Da. The analytes were assayed in the positive mode by quantifying the $[M+Na]+$ ion of α-,β-arteether at m/z 335 and artemisinin at m/z 305.

2.4. Standard and working solutions

Individual standard stock solutions of α-arteether (1 mg ml^{-1}), β-arteether (1 mg ml^{-1}) and internal standard (I.S.) artemisinin (400 μg ml^{-1}) were prepared by accurately weighing required amounts into separate volumetric flasks and dissolving in appropriate volumes of methanol to obtain the required concentrations. Working stock solutions of α-,β-arteether (16 μg ml^{-1}) and I.S. (10 μg ml^{-1}) were prepared from the standard stock solution. The working stock solutions were used to prepare the analytical standards and calibration standards.

Mixed stock solution of α-,β-arteether, I.S. was prepared by transferring 1 ml of the working stock of α-,β-arteether and 2 ml of working stock of I.S. in a 10-ml volumetric flask and the volume made up to result in the highest analytical standard of 1600 ng ml^{-1} of α-,β-arteether and 1000 ng ml^{-1} I.S. Further dilutions were prepared by appropriate dilution ultimately resulting in standards of range of 1600–125 ng ml^{-1} α-,β-arteether and 1000–100 ng ml^{-1} I.S. for the determination of recovery.

Calibration samples of α-,β-arteether from 20 to

320 ng ml^{-1} in normal rat serum were prepared by serial dilution of the working stock solution with normal rat serum so that the volume of the organic phase was less than 2.5% v/v of serum. Quality control (QC) samples at three different concentrations of 20, 80 and 320 ng ml^{-1} in triplicate were prepared separately using a different scheme independent of the calibration samples. The QC samples were used to assess the accuracy and precision of the assay method.

A volume of 5 μl of the working stock solution of the I.S. was added to 200 μl of the serum samples resulting in a concentration of 244 ng ml^{-1} for quantitation. 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 test samples at −30 °C till analysis. The calibration standards were prepared freshly on the day of analysis and analyzed along with the test samples and QC samples.

2.5. Sample preparation

A simple liquid–liquid extraction with 2×2 ml *n*-hexane was used to isolate α-,β-arteether from 0.2 ml fortified rat serum. 0.2 ml of rat serum were placed in 5-ml tubes and 5 μl I.S. (10 μg ml^{-1}) was added and vortex mixed. Hexane (2 ml) was added to each tube and the tubes were vortex mixed for 1 min and centrifuged at 1000 *g* for 5 min. The organic layer was transferred to another tube by freezing the aqueous layer in liquid nitrogen. The aqueous layer was again extracted with 2 ml of hexane and the combined organic layers were evaporated to dryness. The residue was reconstituted in 40 μl of methanol and injected onto the LC–MS system fitted with a 20-μl loop. The calibration curve was obtained by weighted regression ($1/x^2$) of the peak area ratios of α-,β-arteether and I.S. versus concentration with Microsoft Excel version 5.0 on an IBM PC computer.

2.6. Freeze thaw (f–t) stability

QC samples (0.2 ml) at 20, 80 and 320 ng ml^{-1} in duplicates were stored at −30 °C in glass tubes. One set of duplicate samples of each concentration was analyzed immediately after spiking and the other samples after one, two and three f–t cycles. Thawing was achieved by keeping the sample tubes at ambient temperature for 0.5 h. The percent loss of drug during the f–t cycles was determined by comparing the concentrations with that obtained before, immediately after spiking.

3. Method validation

The validation of the LC–MS method included within- and between-assay precision and accuracy studies on three different days and f–t effects. The accuracy and precision were calculated from studies carried out in triplicate at three different concentrations of 20, 80 and 320 ng ml^{-1} in serum and analyzed in each run and three such batches were processed, while the f–t cycle stability samples were processed in duplicates.

3.1. Specificity

The specificity was defined as non-interference in the regions of interest with the endogenous substances, drug metabolites of arteether in the determination of the concentration.

3.2. Lowest limit of detection (LOD) and limit of quantitation (LOQ)

The detection limit of the HPLC assay method (LOD) of α-,β-arteether is the drug quantity in the serum after the sample clean-up corresponding to three times the baseline noise ($S/N>3$). The lowest limit of quantitation (LOQ) was defined as the concentration of the sample, with less than 20% deviation in precision.

3.3. Accuracy and precision

The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples on three different days ($n=27$; three each of low, medium and high concentration). The precision was determined by within- and between-assay %RSD [23]. The accuracy was expressed as %bias:

$$\%\text{Bias} = \frac{(\text{Observed concentration} - \text{nominal concentration}) \times 100}{\text{Nominal concentration}}$$

3.4. Application of the method to biological samples

The assay method described here was applied to study the pharmacokinetics of the Central Drug Research Institute (CDRI) developed i.m. formulation Emal (α-/β-arteether, 30:70) following a single dose of 30 mg kg^{-1} in rats. For i.m.. dose administration, a single 30 mg kg^{-1} dose (0.4 ml kg^{-1}) was injected into the muscle of the rear leg of the rat using a 23-gauge needle. Before the injection, the hair was clipped and the area was cleaned with 70% ethanol solution.

Blood samples (two samples per rat) for pharmacokinetic studies were collected at different time points up to 24-h post dose. Initial samples were collected by cardiac puncture under light ether anesthesia, while terminal samples were collected from inferior venacava. All blood samples were allowed to clot at room temperature for 30 min. Serum was separated by centrifugation at 1000 *g* for 10 min at 4 °C and were stored at −30 °C.

4. Results and discussion

4.1. MS optimization

With the chromatographic conditions established earlier in the laboratory (unpublished data) for the detection using a UV detector, acetonitrile–water (60:40% v/v) was used as the mobile phase to obtain electrospray response of $[M+H]^+$ by flow-injection analysis. No signal for β-arteether could be detected at various cone voltages. Therefore the influence of various concentrations of ammonium acetate additive was explored. During the flow-injection analysis, the best response for α-,β-arteether and artemisinin (I.S.) was obtained with acetonitrile–2.5 m*M* ammonium acetate buffer (pH 5) (60:40% v/v) at a cone voltage optimized to 20 V. The mobile phase at a flow-rate of 1 ml min^{-1} was used with a C_{18} column (Perkin-Elmer, Applied Biosystems, 100×4.6 mm, 5 μm) with a guard column of the same material. Using an UV detector set at λ_{max} 216 nm, the retention times of the analytes were established. The mobile phase and stationary phase conditions were carried onto LC–MS and the analytes were detected for their $[M+NH_4]^+$ adducts. Only α-arteether could be detected and the background showed a distinctive pattern of PEG (44 Da difference: 488.5, 532.5, 576.5, 620.5, 664.6, 708.6). The presence of this pattern in the background was suspected for the suppression of the signals of artemisinin and β-arteether as no such suppression was evident when LC–MS was carried out without the column. RP-18 columns of various brands available in the laboratory were checked for the background under the existing conditions. Spectra showing a 44 Da difference were observed with Pierce (C_{18}, 100×4.6 mm, 5 μm), Discovery (C_{18}, 20×4.6 mm, 5 μm), and Brownlee Perkin-Elmer (Spheri-5, 100×4.6 mm, 5 μm) columns. The intensity of the spectra was highest for Pierce with β-arteether signal being suppressed to 30% of the original signal followed by Brownlee Perkin-Elmer, with signal suppressed to 45% and least for Discovery column, with signal suppressed to 90%. No such pattern was observed with the Ultracarb 5 ODS (30), Phenomenex C_{18} column.

Hence, the Phenomenex column was retained for further work. The effect of the matrix on the ionization of the analytes was explored by direct flow line injection analysis of *n*-hexane extracted blank normal rat serum (200 μl), reconstituted with 40 μl of 1 μg ml^{-1} of the mixture of analytes in mobile phase, acetonitrile, methanol and a combination of methanol and 2.5 m*M* ammonium acetate buffer (pH 5) (60:40% v/v). Surprisingly, $[M+Na]^+$ adducts as intense as $[M+NH_4]^+$ adducts were observed in all the reconstitution solutions and at a cone voltage of 52 V, $[M+Na]^+$ adducts were found to be more intense. The background spectra observed when methanol was used as the reconstitution solution were found to be lower compared to that of acetonitrile. Hence, extracted rat serum (blank) was reconstituted in 40 μl of 1 μg ml^{-1} of α-,β-arteether and I.S. in methanol–2.5 m*M* sodium acetate buffer (pH 5) (60:40% v/v) and explored for the $[M+Na]^+$ and $[M+NH_4]^+$ adducts at a cone voltage of 52 V. Only $[M+Na]^+$ adducts were observed. The molarity of the buffer was optimized to 0.1 m*M* sodium acetate to obtain maximum intensity for the sodium

adducts of α-,β-arteether and I.S. In order to obtain short chromatographic analysis times, a mobile phase of methanol–sodium acetate (0.1 m*M*, pH 5 adjusted with acetic acid) (80:20% v/v) and Ultracarb 5 ODS (30), 30×4.6 mm I.D., 5 μm column were used. A run time of 7 min with artemisinin (I.S.) at 1.22 min, α-arteether at 2.48 min and β-arteether at 4.51 min could be achieved when 200 μl of spiked normal rat serum was extracted with 2 ml of *n*-hexane and reconstituted in 40 μl of methanol and injected onto LC–MS. The extraction efficiency of α-arteether was 90–95% and β-arteether was 80–85%. When 1, 2, 5 and 10% ethyl acetate in hexane, ether, dichloromethane were tried to improve the extracting efficiency, no increase in the extraction efficiency was achieved. Moreover, increase in the polarity of the extracting solvent resulted in endogenous interference. Solid-phase extraction [24] was also tried but neither the extraction efficiency could be increased nor the endogenous interference decreased.

The method was validated using an Ultracarb 5 ODS 20, 30×4.6 mm, 5 μm, Phenomenex. The stationary phase consisted of 22% of carbon compared to 31% in the ODS 30 column and is found to be more suitable for resolving non-polar and hydrophobic compounds. The compounds eluted quickly reducing the run time to 5 min. The retention times of I.S., α-arteether and β-arteether were 1.02, 1.73 and 2.81 min, respectively. There was no significant variation in the retention times of the analytes. The retention times of α-arteether, β-arteether and I.S. throughout the validation were 1.73±0.05, 2.90±0.08 and 1.01±0.04 min, respectively. Furthermore, the scanning mode of detection was useful in detecting two possible new metabolites, which would not have been possible with selected ion monitoring.

4.2. Linearity and calibration standard range

The peak area ratios of α-,β-arteether to I.S. in rat serum varied linearly with concentration over the range tested (20–320 ng ml^{-1}). The calibration model was selected based on the analysis of the data by linear regression with and without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The residuals improved by weighted ($1/x^2$) least-squares regression. Best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ with $1/x^2$ weighting factor; the correlation coefficients (R^2) for α- and β-arteether were 0.997 and 0.998, respectively.

4.3. Specificity

LC–MS analysis of the blank serum samples showed no endogenous peak interference with the quantification of α-,β-arteether and its respective I.S. Representative chromatograms of blank rat serum fortified with α-, β-arteether with I.S. and extracted blank rat serum are shown in Fig. 2b,c, 2e,f and 2h,i, respectively.

4.4. Sensitivity

The LOD determination demonstrated that all the analytes gave a signal-to-noise ratio of 3:1 and above for 5 ng ml^{-1} extracted and injected. The limit of quantification (LOQ) for α-,β-arteether using 200 μl of normal rat serum was established at a concentration of 20 ng ml^{-1} for both the analytes.

4.5. Assay validation

4.5.1. Recovery

The recovery of the I.S. from all the extracted calibration and QC samples during validation was 73.90±6.9%. The average recoveries for α-,β-arteether over the concentration range of 20–320 ng ml^{-1} is shown in Table 1.

4.5.2. Accuracy and precision

The overall percent bias and precision at the three concentrations are presented in Table 2. The result show that the analytical method is accurate and the bias is within the acceptance limits of ±20% at low concentration and ±15% at all the concentration levels studied. The within-day and between-day %CVs for α- and β-arteether were found to be less than 10%.

4.6. Stability

4.6.1. Bench top stability

There was no significant difference (<±15%) between the responses of standards at time zero and

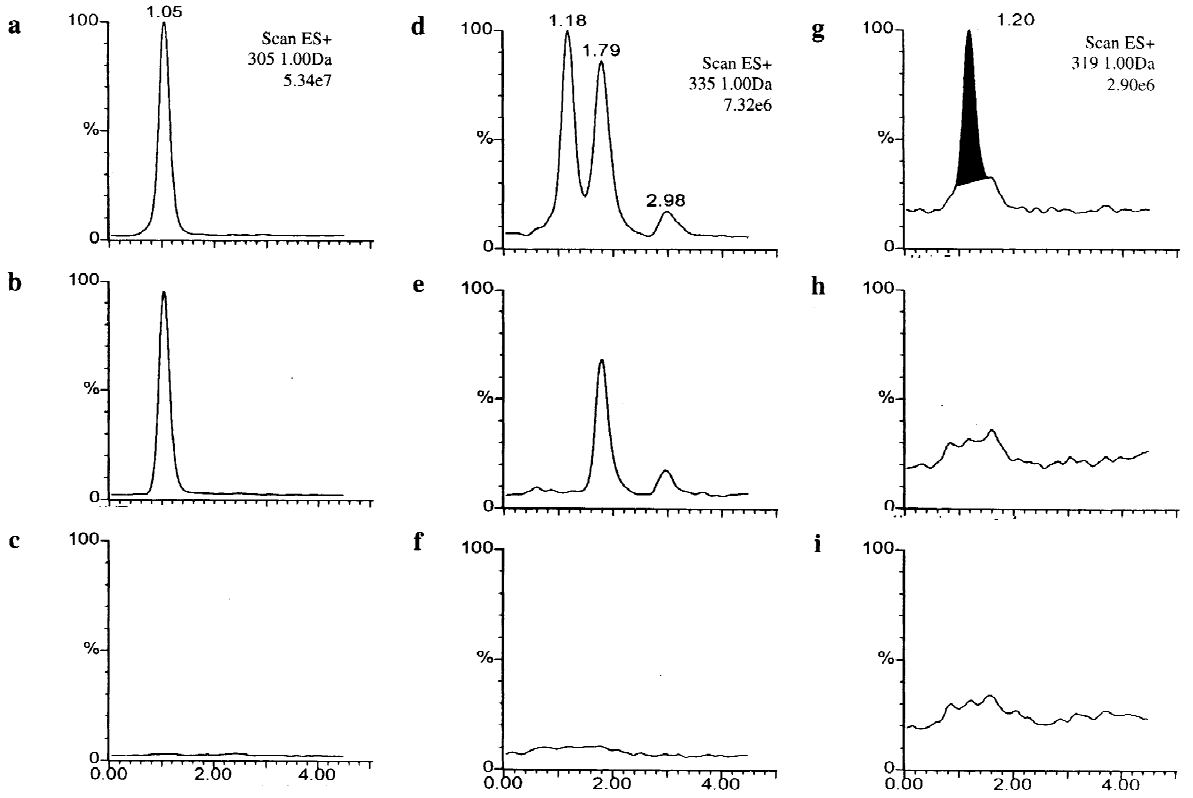

Fig. 2. Chromatogram of (a) artemisinin in test sample (120 min); (b) normal rat serum fortified with α-,β-arteether (80 ng/ml) and artemisinin (250 ng/ml); (c) blank normal rat serum; (d) α-,β-arteether in test sample (120 min); (e) normal rat serum fortified with α-,β-arteether (80 ng/ml) and artemisinin (250 ng/ml); (f) blank normal rat serum; (g) test sample (120 min) at *m*/*z* 319; (h) normal rat serum fortified with α-,β-arteether (80 ng/ml) and artemisinin (250 ng/ml); (i) blank rat serum at *m*/*z* 319.

after 12 h in terms of %CV (4.8%) for both α- and β-arteether, indicating the stability of α-,β-arteether at room temperature over 12 h. Moreover, the analytes were found to be stable after reconstitution for at least 12 h at 4 °C as the %CV at all the three concentration levels was less than 10%.

Table 1
Mean recoveries of α- and β-arteether from spiked rat serum

Concentration (ng/ml)	Absolute recovery (mean±SD, $n=3$) (%)	
	α-Arteether	β-Arteether
20	96.2±2.84	71.3±17.1
40	93.3±0.90	69.8±10.3
80	99.8±6.46	74.8±10.8
160	93.3±10.84	71.7±11.5
320	88.4±0.68	79.6±2.42

4.6.2. Freeze–thaw stability in serum

The deviation observed after one, two and three f–t cycles was within ±10% at LOQ for both α- and β-arteether indicating freeze–thaw stability (Fig. 3a,b).

4.7. Application of the method to biological samples

The method was applied to investigate the pharmacokinetics of α-,β-arteether in male Sprague–Dawley rats following the administration of a single i.m. dose of 30 mg kg^{-1} Emal. Serial blood samples were collected post i.m. dose and the serum was collected and stored at −30 °C till further analysis. The method was sensitive enough to follow α-,β-arteether up to 24-h post dose. Typical representative

Table 2
Accuracy and precision of α- and β-arteether in rat serum

Analyte	Conc. (ng/ml)	%Bias		%RSD	
		Intra batch	Inter batch	Intra batch	Inter batch
α-arteether	20	6.0	3.3	2.6	10.7
	80	2.4	2.8	3.2	6.4
	320	−8.8	−9.3	1.9	4.0
β-arteether	20	3.9	2.6	9.6	7.2
	80	−2.3	−2.5	4.4	6.1
	320	3.5	2.9	3.1	8.1

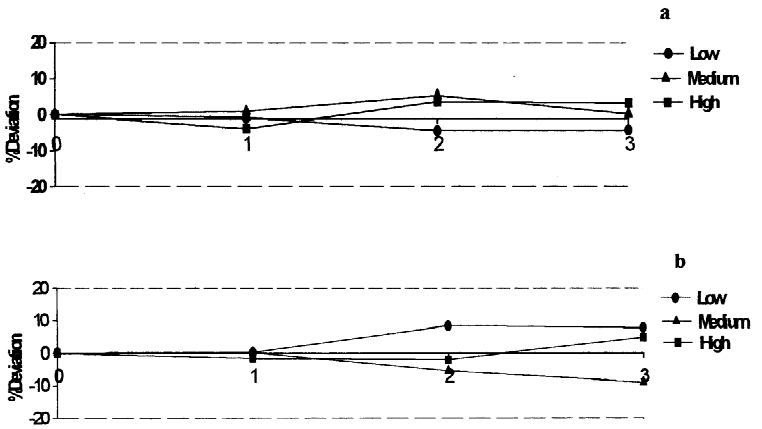

Fig. 3. Freeze–thaw cycle stability; (a) α-arteether; (b) β-arteether.

chromatograms of blank and treated rat serum are given in (Fig. 2a,c,d,f,g,i). In Fig. 2b,e and h, normal rat serum is fortified with α-,β-arteether and artemisinin. At m/z 305, only artemisinin is seen (Fig. 2b). Fig. 2e exhibits both α-,β-arteether at m/z 335. Whereas, there is no interference in the region of the metabolite (m/z 319) with either of the analytes in Fig. 2h. A new peak eluted at 1.18 min at $m/z = 335$ (Fig. 2d). This peak was found to follow the profile of the parent compounds and was not found in 0-min samples. Another possible metabolite at $m/z = 319$ ($296 + Na^+$), corresponding to deoxyarteether ($m/z =$ 296) was also detected in the test samples (Fig. 2g). The mean serum concentration–time profile of α-,β-arteether is shown in Fig. 4. A discontinuous release profile of the parent compounds from the muscle can be seen and moreover levels were detected for as long as 24 h.

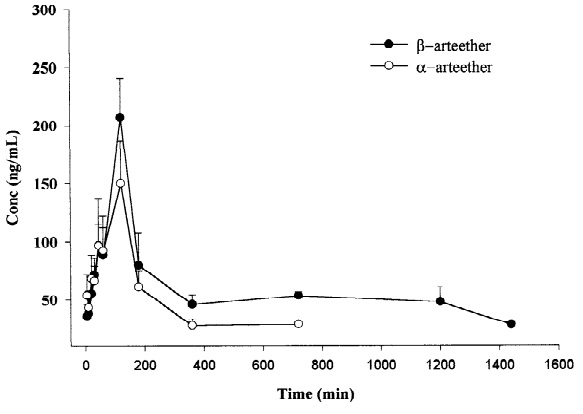

Fig. 4. Concentration–time profiles of α-arteether and β-arteether following i.m. administration of 30 mg/kg α-,β-arteether (Emal) in rats.

5. Conclusion

The liquid–liquid extraction method gave high and consistent recoveries for α- and β-arteether and I.S. and provided clean extracts. HPLC–MS with electrospray ionization in positive ion mode of detection appeared to be a sensitive and selective method for the determination as reported earlier by electrochemical detection, but with reduced (five times) volume of serum requirement. Moreover, the method also allows for the detection of new metabolites, which have not been reported earlier by assay methods using electrochemical detectors. Hence the method is highly suitable for carrying out preclinical pharmacokinetic studies in rats.

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