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Liquid chromatography–tandem mass spectrometry for the simultaneous quantitation of artemether and lumefantrine in human plasma: Application for a pharmacokinetic study

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ABSTRACT

A liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) method for the simultaneous quantitation of artemether and lumefantrine in human plasma was developed and validated. Artesunate was used as an internal standard (IS). The analytes were extracted by a protein precipitation procedure and separated on a reversed-phase Zorbax SB-Ciano column with a mobile phase composed of methanol and 10 mM aqueous ammonium acetate containing 0.2% (v/v) acetic acid and 0.1% (v/v) formic acid. Multiple reaction monitoring was performed using the transitions m/z 316 → m/z 267, m/z 530 → m/z 348 and m/z 402 → m/z 267 to quantify artemether, lumefantrine and artesunate, respectively. Calibration curves were constructed over the range of 10–1000 ng/mL for artemether and 10–18,000 ng/mL for lumefantrine. The lower limit of quantitation was 10 ng/mL for both drugs. The mean R.S.D. values for the intra-run precision were 2.6% and 3.0% and for the inter-run precision were 3.6% and 4.6% for artemether and lumefantrine, respectively. The mean accuracy values were 102.0% and 101.2% for artemether and lumefantrine, respectively. No matrix effect was detected in the samples. The validated method was successfully applied to determine the plasma concentrations of artemether and lumefantrine in healthy volunteers, in a one-dose pharmacokinetic study, over the course of 11 days.

1. Introduction

Due to the widespread resistance of *Plasmodium falciparum* to conventional antimalarial drugs, many countries are facing problems regarding the treatment of uncomplicated malaria [1]. The main therapy now recommended by the World Health Organization is artemisinin-based combination therapy (ACT), a combination of an artemisinin derivative and another structurally-unrelated and slowly-eliminated antimalarial [2]. Artemether–lumefantrine (20 + 120 mg) (Fig. 1) is the most common ACT used in malaria endemic areas [3].

A quantitative determination of artemether and lumefantrine in plasma is essential in order to evaluate the bioavailability and pharmacokinetics of these co-administrated antimalarials. Previous studies have suggested that the area under the plasma lumefantrine concentration–time curve was the principal determinant of curing malaria [4], and artemether formulations with a high bioavailability are considerably important to their clinical efficacy [5].

Some methods have been reported for determining the presence of either artemether [5–10] or lumefantrine [4,11–16] in human plasma. McGready et al. [17] evaluated the pharmacokinetics of both drugs in pregnant women; however, the artemether and lumefantrine analyses were carried out separately. César et al. [18] developed an LC-UV method for the simultaneous quantitation of artemether and lumefantrine in fixed-dose combination tablets. Nevertheless, ultraviolet detection is not adequate for artemether quantitation in a biological matrix due to its low sensitivity and selectivity. The majority of artemether quantitation in plasma is performed by electrochemical detection, and some works detected this drug by mass spectrometry using atmospheric pressure chemical ionization (APCI) [9,10] or electrospray ionization (ESI) [19,20]. Hodel et al. [19] developed an HPLC–ESI–MS/MS method for the quantitation of 14 antimalarial drugs in human plasma, including artemether and lumefantrine. However, the method developed in our study involves a shorter analysis time, the absence of the drying

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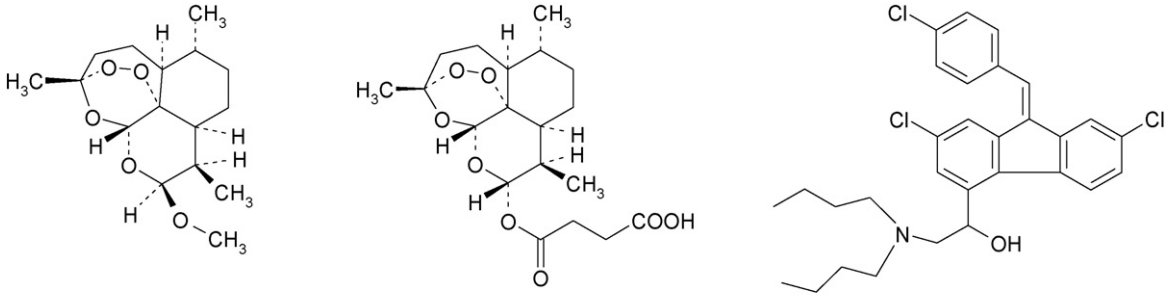

Fig. 1. Chemical structures and molar masses of artemether, artesunate (IS) and lumefantrine.

step in sample preparation and a shorter chromatographic run time. In addition, this is the first report providing the simultaneous quantitation of artemether and lumefantrine with a pharmacokinetic application.

Hence, the aim of this work was to develop and validate an HPLC–ESI–MS/MS method for the simultaneous quantitation of artemether and lumefantrine in human plasma. The method was applied to a pharmacokinetic study in healthy volunteers who received the fixed-dose combination tablets.

2. Experimental

2.1. Chemicals and reagents

Artemether, lumefantrine and artesunate (the internal standard) reference standards were purchased from Dafra Pharma (Turnhout, Belgium). Coartem® (Novartis, Basel, Switzerland) tablets were kindly donated by the Brazilian Health Ministry. Ultrapure water was obtained from a Millipore system (Bedford, MA, USA). Methanol (HPLC grade) was purchased from Tedia (Fairfield, OH, USA) and acetic acid, formic acid and ammonium acetate (analytical grade) were from J. T. Baker (Phillipsburg, NJ, USA).

2.2. Instrumentation and analytical conditions

The HPLC–ESI–MS/MS analyses were carried out on a Waters system (New Castle, DE, USA), composed of a 1525 μ binary pump, a 2777 sample manager, a TCM/CHM column oven and a Quattro LC triple quadrupole mass spectrometer, equipped with an electrospray ion source. MassLynx v.4.1 software was used for data acquisition and analysis. LC separation was performed on a Zorbax SB-Ciano column (150 mm × 4.6 mm i.d.; 5 μm particle size) from Agilent (Santa Clara, CA, USA) with a similar Zorbax SB-Ciano security guard column (12.5 mm × 4.6 mm, 5 μm), and it was maintained at 30 °C. The mobile phase consisted of (A) methanol and (B) 10 mM aqueous ammonium acetate containing 0.2% (v/v) acetic acid and 0.1% (v/v) formic acid. The mobile phase was delivered using a linear gradient elution program: 60% methanol (solvent B) at 0 min, 100% B at 7 min and 60% B from 7 to 9 min (re-equilibration step), at a flow rate of 1 mL/min. The injection volume was 50 μL, aiming to optimize the drug signals.

Mass spectrometric detection was performed using an electrospray ion source in the positive ionization mode. Nitrogen was used as both the nebulizing and the desolvation gas and argon was used as the collision gas (1.8×10^{-3} to 2.0×10^{-3} mbar). The ion source parameters were: capillary 3.5 kV, extractor 2 V, RF lens 0.5 V, source temperature 80 °C, desolvation temperature 150 °C, artemether and artesunate cone 15 V and lumefantrine cone 45 V. The multiplier was set at 650 V. The collision energies were optimized at 10 eV for artemether and artesunate and 40 eV for lumefantrine. Selected reaction monitoring (SRM) was employed for the data acquisition. The precursor ions of artemether and artesunate were the ammonium adduct $[M+NH_4]^+$, and $[M+H]^+$ was the precursor ion of lumefantrine. The MRM fragmentation transitions were set in the MS program in the following order: m/z 316 → m/z 267, m/z 530 → m/z 348 and m/z 402 → m/z 267 for artemether, lumefantrine and artesunate, respectively. The scan dwell time was set at 0.35 s for each channel.

2.3. Preparation of standard solutions

Stock solutions of artemether (250 μg/mL) and artesunate (500 μg/mL) were prepared by dissolving the accurately weighed reference substance in methanol. A lumefantrine stock solution (2500 μg/mL) was prepared in a similar way using methanol and glacial acetic acid (100:2) as a solvent. The working solution of artesunate (IS) was prepared by diluting the stock solution with methanol to a final concentration of 5 μg/mL. All of the stock solutions were prepared immediately before use.

2.4. Preparation of calibration and QC samples

The working solutions containing both artemether and lumefantrine were prepared using serial dilutions of the stock solutions with methanol and water (50:50, v/v). Seven calibration samples were prepared by spiking the appropriate amounts of these working solutions into blank plasma obtained from healthy volunteers. The concentration of the calibration samples in plasma were 10, 50, 100, 250, 500, 750 and 1000 ng/mL for artemether and 10, 200, 2000, 6000, 10,000, 14,000 and 18,000 ng/mL for lumefantrine. Quality control (QC) samples in plasma were prepared in a similar way, at high, middle and low concentrations: 30, 400 and 800 ng/mL for artemether and 30, 7500 and 15,000 ng/mL for lumefantrine.

2.5. Sample preparation

A 50 μL aliquot of the IS solution (5 μg/mL of artesunate in methanol) was added to 250 μL of the plasma sample. The sample was vortex mixed for 30 s. A 450 μL aliquot of glacial acetic acid 0.5% (v/v) in methanol was added, and the sample was vortexed for 40 s. The samples were then centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a vial and a 50 μL aliquot was injected into the chromatographic system.

2.6. Method validation

The validation process was carried out according to Guidance for Industry–Bioanalytical Method Validation, recommended by the US Food and Drug Administration [21].

Selectivity of the method was evaluated by assaying human blank plasma samples from six different donors, including one lipemic and one hemolyzed plasma sample. These samples were compared to those containing artemether or lumefantrine at the lower limit of quantitation (LLOQ) or artesunate at 1000 ng/mL. In

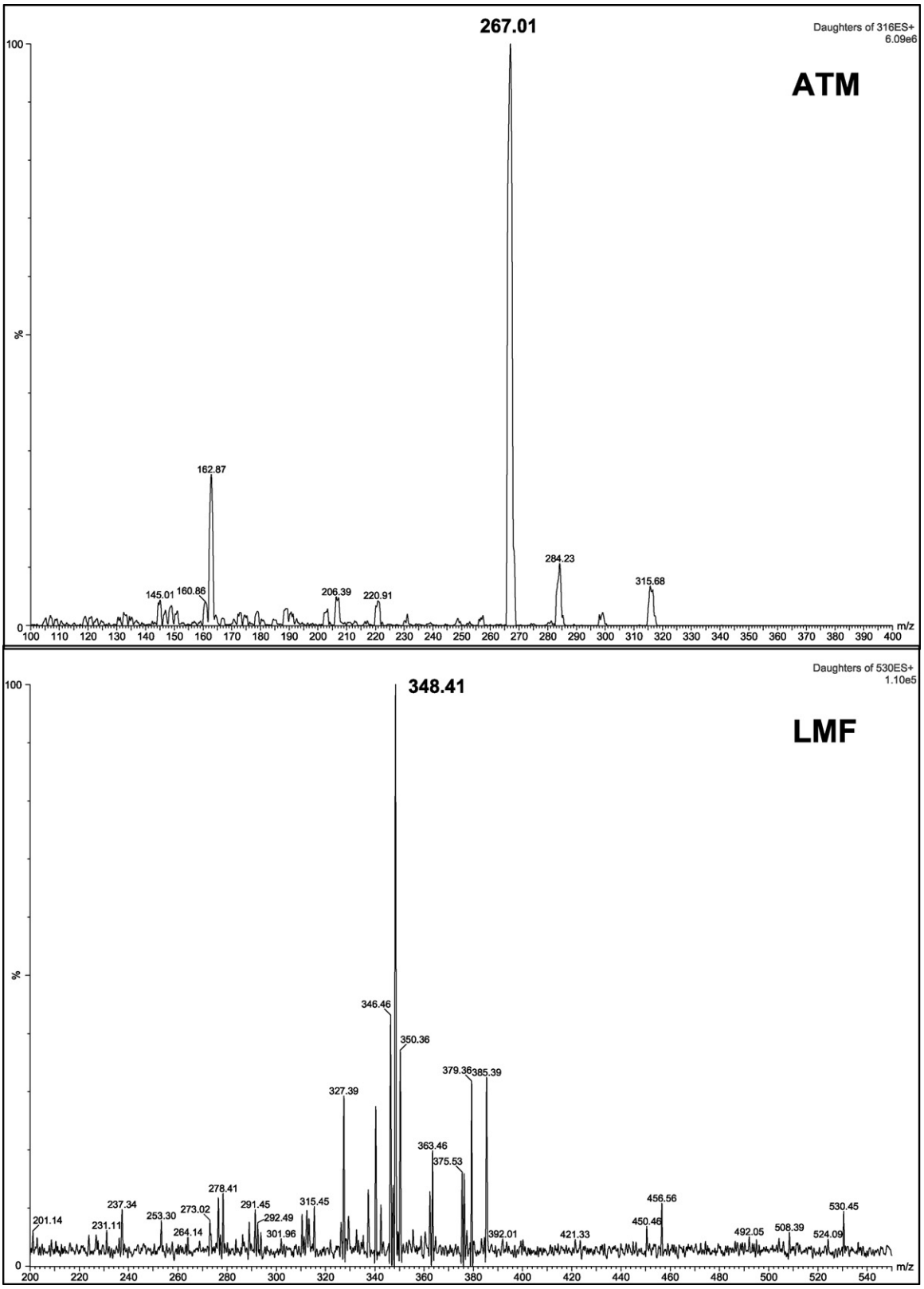

Fig. 2. Product ion mass spectra of artemether (ATM) and lumefantrine (LMF). The product ions monitored were *m*/*z* 267 for artemether and *m*/*z* 348 for lumefantrine.

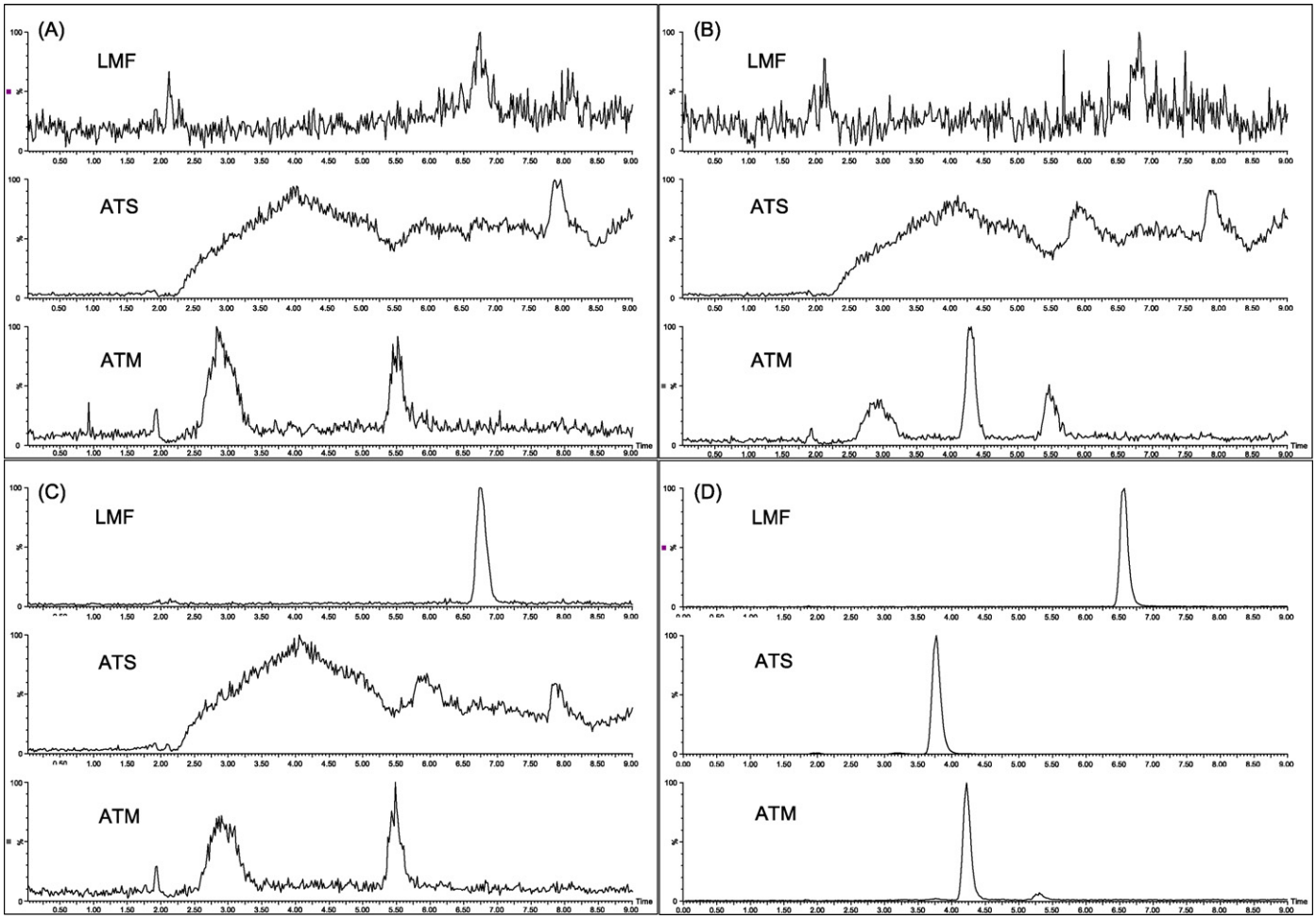

Fig. 3. MRM chromatograms of (A) blank plasma, (B) blank plasma spiked with artemether (ATM) at LOQ (10 ng/mL), (C) blank plasma spiked with lumefantrine (LMF) at LOQ (10 ng/mL) and (D) volunteer plasma collected 3 h after oral administration of a single dose of 80 mg of artemether and 480 mg of lumefantrine.

addition, plasma samples spiked with caffeine (1 μg/mL), dexchlorpheniramine (76 ng/mL), metamizole (5 μg/mL), acetaminophen (20 μg/mL) or dihydroartemisinin, the main artemether metabolite (1 μg/mL) were also evaluated to ensure that there was no interference in the method.

Linearity was assessed by seven-point calibration curves in human plasma in duplicate on three consecutive days. The curves were constructed by plotting the peak area ratio of each antimalarial to the IS versus the concentration of artemether or lumefantrine. The concentration range evaluated for artemether was 10–1000 ng/mL and for lumefantrine was 10–18,000 ng/mL. The curves were evaluated by residuals and fitted by weighted linear regression (artemether) or weighted quadratic regression (lumefantrine). The LLOQ was established as the lowest concentration of the calibration curve at which the precision was within 20% and the accuracy was within 20% by means of the analyses of five replicates. In addition, the analyte response at this concentration should be at least five times the baseline noise.

To evaluate the precision and accuracy of the method, QC samples at three concentration levels (30, 400 and 800 ng/mL for artemether and 30, 7500 and 15,000 ng/mL for lumefantrine) were analyzed in six replicates on three different days. Intra-run and inter-run precision were calculated and expressed as relative standard deviations (R.S.D.%).

The extraction recovery of the method was determined by comparing the peak areas obtained from the plasma samples with those of directly injected standards at the same concentration. It was evaluated by analyzing six replicates containing 30, 400 and 800 ng/mL of artemether and 30, 7500 and 15,000 ng/mL of lumefantrine. The recovery of the IS was determined in a similar way at the work concentration (1000 ng/mL of artesunate).

The matrix effect was evaluated to verify whether the potential ion suppression or enhancement due to the co-elution matrix components existed in the analysis. The peak areas of artemether, lumefantrine and the IS from the spike-after-protein precipitation samples were compared to those of the standard solutions in the mobile phase at the same concentrations. This experiment was carried out with blank plasma samples from six different donors at low and high QC concentrations of artemether and lumefantrine.

The stability of the analytes in plasma was evaluated under a variety of storage and handling conditions using the low and high QC samples in six replicates. Freeze–thaw stability was evaluated after three complete free/thaw cycles (−70 to 23 °C) on consecutive days. Short-term temperature stability was assessed by analyzing samples that were kept at ambient temperature (23 °C) for 6 h. Long-term stability was performed using plasma samples that were stored at −70 °C for 1, 2 and 3 months. To evaluate the post-preparative stability, QC samples were extracted and kept in the autosampler (4 °C) for 10 h before the injection. The stabilities of the work solutions of artemether, lumefantrine and the IS at 4 and 23 °C for 6 h were also evaluated. The analytes were consid-

ered stable when 85–115% of the initial concentrations were found.

2.7. Application to a clinical pharmacokinetic study

The validated method was used to determine the plasma concentration of artemether and lumefantrine in a pharmacokinetic study using the fixed-dose combination tablets. Five healthy volunteers, under fasting conditions, received a single oral dose of 80 mg of artemether and 480 mg of lumefantrine, corresponding to four tablets of the fixed-dose combination, with 200 mL of milk. The blood samples (10 mL) were collected into heparinized tubes at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 72, 120, 168, 216 and 264 h after drug administration. Plasma samples were obtained by centrifugation at 2000 rpm for 10 min and frozen at −70 °C until the analyses. The study protocol was approved by the Ethics Committee of the Universidade Federal de Minas Gerais.

3. Results and discussion

3.1. Conditions for MS/MS

Initially, the ionization and detection of the analytes in the mass spectrometer were evaluated by atmospheric pressure chemical ionization (APCI), according to the previous works of Souppart et al. [9] and Shi et al. [10] for artemether. However, this ion source provided an artemether precursor ion $[M+H]^+$ at a considerably low intensity; also, lumefantrine could not be adequately detected. Xing et al. [22] and Sabarinath et al. [23] performed the quantitation of artemisinin derivatives by LC–MS/MS using electrospray ionization by monitoring the ammonium adduct $[M+NH_4]^+$ as the precursor ion. Hence, 10 mM ammonium acetate buffer was included in the mobile phase and the precursor ions $[M+NH_4]^+$ of artemether m/z 316 (298 g/mol of artemether + 18 g/mol of ammonium) and artesunate m/z 402 (384 g/mol of artemether + 18 g/mol of ammonium) could be properly detected. The optimized conditions showed to be adequate for the lumefantrine ionization as well, with the detection of the precursor ion $[M+H]^+$ at m/z 530. At a collision energy of 10 eV, artemether and artesunate (IS) presented the same intense product ion at m/z 267. For lumefantrine, the major product ion was m/z 348, at a collision energy of 40 eV. The product ion mass spectra of artemether and lumefantrine are presented in Fig. 2.

3.2. Conditions for HPLC

The use of 10 mM ammonium acetate buffer in the mobile phase was essential for detection of the ammonium adduct $[M+NH_4]^+$ of artemether and artesunate. The mobile phase acidification with acetic acid and formic acid was important to ensure an adequate lumefantrine peak shape and to promote the ionization of the analytes in the positive mode. Initially, an isocratic elution condition was evaluated (10 mM ammonium acetate buffer:methanol–20:80). However, interference of the matrix components was detected, resulting in high recovery percentages due to an ionization enhancement of the analytes. Thus, a linear gradient elution program was employed, using 60% methanol (solvent B) at 0 min and 100% B at 7 min, so that no matrix effect was verified in this optimized condition. The column re-equilibration time was 2 min, with 60% methanol, so that the total run time was 9 min. The retention times were about 3.8, 4.2 and 6.7 min for artesunate (IS), artemether and lumefantrine, respectively. The chromatograms obtained with this developed method are shown in Fig. 3.

3.3. Sample extraction

Liquid–liquid extraction was initially considered for sample preparation. However, compared to protein precipitation, this procedure proved to be considerably time-consuming and laborious, resulting in a reduction of precision and recovery rate of the assay. The developed protein precipitation procedure is simple, robust and provided high recovery rates for all analytes, resulting in a fast and easily-handled analysis.

3.4. Method validation

No significant interference was detected at the retention times of the analytes in the six different blank plasma chromatograms (Fig. 3). The plasma samples spiked with caffeine, dexchlorpheniramine, metamizole, acetaminophen or dihydroartemisinin did not present response at the ion transitions selected for the analytes quantitation.

The artemether calibration curves were shown to be linear over the range of 10–1000 ng/mL, with a regression coefficient higher than 0.998. A typical artemether standard curve was $y = 8.638 \times 10^{-4}x + 34.456 \times 10^{-4}$, with a weighted factor $1/x$. For lumefantrine, a linear regression model was evaluated first; however, a weighted $(1/x)$ quadratic regression model provided a better fit for the validation data. A typical lumefantrine curve was $y = -6.3343 \times 10^{-9}x^2 + 5.588 \times 10^{-4}x + 26.244 \times 10^{-4}$, with regression coefficients > 0.997. The residuals had no tendency of variation with concentration for both artemether and lumefantrine. The obtained LLOQ was 10 ng/mL for both drugs, with a precision of 10.4% and 12.8% in terms of R.S.D. and an accuracy of 100.4% and 93.4% for artemether and lumefantrine, respectively.

The intra-run and inter-run precision and accuracy were calculated by analyzing six replicates of QC samples at three concentration levels on three different days. The obtained data are shown in Table 1. The mean R.S.D. values in the intra-run precision were 2.6% and 3.0%, and the inter-run precision values were 3.6% and 4.6% for artemether and lumefantrine, respectively. The mean accuracy values were 102.0% and 101.2% for artemether and lumefantrine, respectively. These data indicated reproducible LC–MS/MS results and that the assay was accurate and reliable.

The mean recovery rates of artemether and lumefantrine ($n = 18$), determined at three concentrations, were 85.9% and 82.1%,

Table 1
Precision, accuracy and recovery data for the assay of artemether and lumefantrine by LC–MS/MS.

Validation parameters	Artemether quality control concentration (ng/mL)			Lumefantrine quality control concentration (ng/mL)		
	30	400	800	30	7500	15,000
Precision (R.S.D.%)						
Intra-run ($n = 6$)	3.4	2.8	1.6	5.1	2.1	1.9
Inter-run ($n = 18$)	5.2	3.3	2.4	5.9	5.5	2.4
Accuracy (%)						
Intra-run ($n = 6$)	97.1	98.3	100.7	106.4	102.7	103.5
Inter-run ($n = 18$)	103.3	100.2	102.6	103.6	97.6	102.4
Recovery (%) ($n = 6$)	83.4	85.5	88.7	81.4	83.2	81.8

Table 2
Stability data for the assay of artemether and lumefantrine by LC–MS/MS.

Compound	Spiked amount (ng/mL)	% loss/gain in the stability study ($n = 6$)			
		Freeze–thaw	Short-term	Long-term	Post-preparative
Artemether	30	+5.1	+10.3	+5.4	−1.7
	800	−0.2	−3.9	−3.9	−3.5
Lumefantrine	30	−1.8	+3.6	−8.9	+10.7
	15,000	−2.5	−3.8	−0.1	+1.7

respectively. The recovery of the IS was shown to be 86.7% ($n = 18$). The recovery data are shown in Table 1.

The matrix effect was evaluated by comparing the mean peak areas of artemether, lumefantrine and IS from the spike-after-protein precipitation samples with those of the standard solutions in the mobile phase. The observed variation did not exceed the range 85–115%, so that in the present LC–MS/MS method, the matrix effects for all analytes were not significant.

The results of the stability experiments (Table 2), considering the analyte/IS ratio, showed that the artemether and lumefantrine plasma samples were stable for up to 6 h at 23 °C, for 3 months at −70 °C, for 10 h after extraction in the autosampler and after three complete freeze/thaw cycles on consecutive days, as the R.E. values were within 15% for both analytes, at low and high concentrations. Samples spiked with the IS were stable for at least 6 h at 23 °C.

3.5. Application to a clinical pharmacokinetic study

The validated method was applied to a pharmacokinetic study in healthy volunteers. The sensitivity and specificity of the method showed to be adequate for accurately characterizing the pharmacokinetics of artemether and lumefantrine. The mean plasma concentration–time curves of artemether and lumefantrine are shown in Fig. 4. The main pharmacokinetic parameters of both drugs were calculated. The mean C_{max} for artemether, 57.37 ng/mL, was reached 1.9 h (T_{max}) after drug administration, whereas for lumefantrine a C_{max} of 1979.95 ng/mL was reached after 5.8 h. The mean values of AUC_{0-t} obtained were 156.20 and 40664.64 ng h/mL for artemether and lumefantrine, respectively. The mean values of $AUC_{0-\infty}$ were 184.70 and 42870.75 ng h/mL for artemether and lumefantrine, respectively. The elimination half-life of artemether and lumefantrine were 1.8 and 76.5 h, respectively. These results are consistent with those previously reported [4,10,24], which demonstrate a rapid absorption and elimination of artemether, while lumefantrine presented a long elimination half-life and could be detected in the volunteers' plasma for up to 11 days.

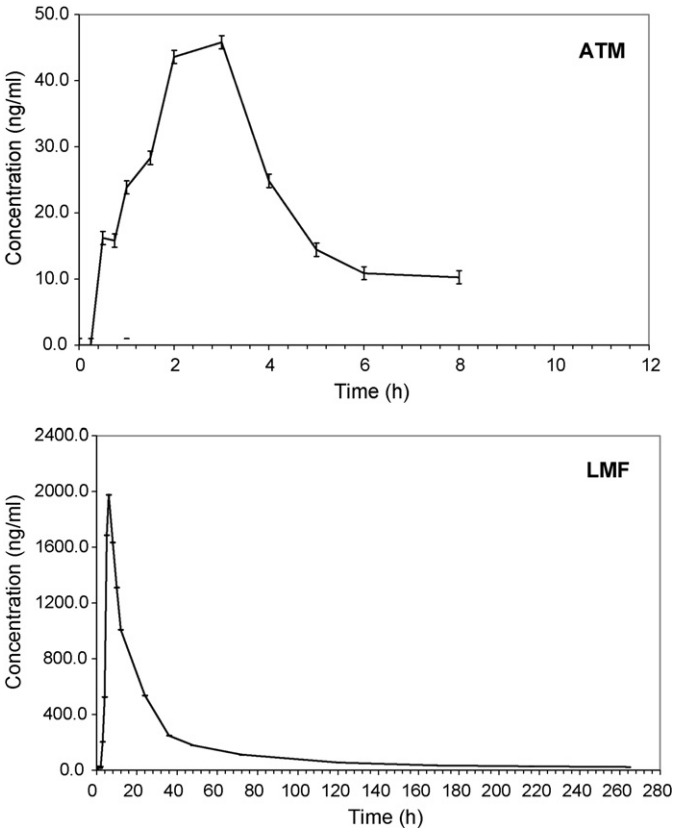

Fig. 4. Plasma concentrations of artemether (ATM) and lumefantrine (LMF) after oral administration of a single dose of 80 mg of artemether and 480 mg of lumefantrine. Bars represent the variation of a ±2 standard error value regarding the mean concentrations.

4. Conclusion

The developed HPLC–ESI–MS/MS method allowed the simultaneous quantitation of artemeter and lumefantrine in human plasma and provided simple and rapid analyses, as well as sensitive and reliable results. Thus, this method showed to be suitable for routine high-throughput analyses and may be successfully applied to pharmacokinetic and bioequivalence studies of at least the single dose evaluated in the present work (80 mg of artemether and 480 mg of lumefantrine) in human subjects.

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