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# Determination of β-artemether and its main metabolite dihydroartemisinin in plasma employing liquid-phase microextraction prior to liquid chromatographic–tandem mass spectrometric analysis

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ABSTRACT

A method for the determination of artemether (ART) and its main metabolite dihydroartemisinin (DHA) in plasma employing liquid-phase microextraction (LPME) for sample preparation prior to liquid chromatography–tandem mass spectrometry (LC–MS–MS) was developed. The analytes were extracted from 1 mL of plasma utilizing a two-phase LPME procedure with artemisinin as internal standard. Using the optimized LPME conditions, mean absolute recovery rates of 25 and 32% for DHA and ART, respectively, were achieved using toluene–*n*-octanol (1:1, v/v) as organic phase with an extraction time of 30 min. After extraction, the analytes were resolved within 5 min using a mobile phase consisting of methanol–ammonium acetate (10 mmol $L^{-1}$, pH 5.0, 80:20, v/v) on a laboratory-made column based on poly(methyltetradecylsiloxane) attached to a zirconized-silica support. MS–MS detection was employed using an electrospray interface in the positive ion mode. The method developed was linear over the range of 5–1000 ng $mL^{-1}$ for both analytes. Precision and accuracy were within acceptable levels of confidence (<15%). The assay was applied to the determination of these analytes in plasma from rats treated with ART. The two-phase LPME procedure is affordable and the solvent consumption was very low compared to the traditional methods of sample preparation.



## 1. Introduction

The herb *Artemisia annua* L. (Asteraceae), also known as *qinghao*, has been used in the Chinese medicine for the treatment of malaria for more than 2000 years. In the early seventies, several research groups carried out studies employing medicinal plants reported in Chinese traditional practice in an effort to discover novel therapeutic agents. The extracts belonging to *qinghao*, which displayed highly effective antimalarial activity, stood out from the others tested [1]. Thereafter, the compound responsible for this property was isolated and characterized, receiving the name artemisinin (ATM). Since the discovery of ATM, several derivatives have been developed in order to overcome the problems of solubility of this compound, thereby permitting it to be applied in the treatment of malaria [2]. ATM and its derivatives are sesquiterpene lactones containing an endoperoxide bridge in their structures (Fig. 1). Although the exact mechanism of anti-plasmodial activity of this class of compounds has not been fully understood, some studies have suggested the mediation of free radicals derived from the endoperoxide linkage in this process [3]. Moreover, among several desirable properties as antimalarial drugs, ATM and its derivatives lack pronounced toxicity and show rapid onset of action, reducing parasitemia in a few hours [4].

β-Artemether (ART, Fig. 1), one of the ATM derivatives clinically employed in the therapy of malaria, is an oil-soluble methyl ether of ATM and has been used in oral, rectal and intramuscular formulations [2]. Furthermore, the semi-synthetic ART is more potent than ATM *in vitro*, thus making this agent an useful option in the treatment of infections caused by multidrug-resistant strains of *Plasmodium falciparum* [4]. ART is hepatically biotransformed by CYP 2B6 to dihydroartemisinin (DHA, Fig. 1), which is the main metabolite and also presents antimalarial properties both *in vitro* and *in vivo* [5]. Some authors have claimed that ART may be a prodrug of DHA and, therefore, the quantification of this compound in biological fluids is needed in therapeutic monitoring [6].

Several efforts have been made to develop analytical methods devoted to the determination of ART and DHA in biological

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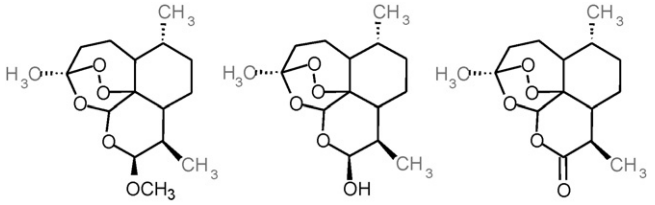

**Fig. 1.** Structures of ART (*left*), DHA (*middle*) and ATM (*right*), used as internal standard.

matrices, which is frequently hampered by difficulties owing to poor detection, thermal unstability, lack of groups amenable to derivatization as well as the low levels of these analytes commonly observed in these samples [7]. Even so, some methods have been described in the literature. Initially, the methods relied on the separation and quantification of the degradation products of the drug and its metabolite in plasma using HPLC with UV detection [8,9]. Nevertheless, the application of these methods in pharmacokinetic studies is limited due to unsatisfactory detectivity. Later, Mohamed et al. [10] utilized this approach to analyze ART and DHA in plasma using gas chromatography as the analytical technique. HPLC with electrochemical detection was the most used technique for the determination of these analytes in the last decade since the endoperoxide bridge suffers reduction and this phenomenon may be employed for detection purposes [11–14]. In spite of adequate selectivity and the low limits of quantification that were reported, the utilization of these methods on regular basis is constrained owing to the rigorous procedures for deoxygenation of the system and samples. More recently, HPLC with mass spectrometric detection (HPLC–MS) using either electrospray (ESI) or atmospheric pressure chemical ionization (APCI) has also been described for the analysis of ART and DHA in plasma [15–18].

Liquid-phase microextraction (LPME) employing porous capillary membranes is an attractive technique for sample preparation prior to chromatographic or electrophoretic analysis [19]. LPME, a miniaturized approach similar to conventional liquid–liquid extraction (LLE), relies on the diffusion of analytes from aqueous samples to a thin layer of organic solvent held in the pores of the membrane and then to a small volume of acceptor phase supported in the lumen of the cylindrical membrane, which may be of either organic (two phases) or aqueous nature (three phases) [20]. This technique has been succesfully applied for the extraction of some antimalarial drugs and their metabolites in plasma [21–23].

Since all methods described in the literature for the analysis of ART and DHA have used traditional techniques of sample preparation, the aim of this study was to develop and validate a method employing an affordable and solvent-minimizing LPME procedure for the extraction of these analytes from plasma followed by LC–MS–MS analysis.

## 2. Experimental

### 2.1. Chemicals and reagents

ART and DHA were generously supplied by Dafra Pharma NV (Oud-Turnhout, Belgium). ATM, utilized as internal standard (IS), was acquired from Sigma (St. Louis, MO, USA). Methanol and ammonium acetate salt, used for the preparation of the mobile phases, were purchased from Merck (Darmstadt, Germany). Butyl ether, hexyl acetate and *n*-octanol were obtained from Sigma, while toluene was acquired from Mallinckrodt Baker (Phillipsburg, NJ, USA). Purified water was obtained from a Milli-Q-system (Millipore, Milford, MA, USA). All chemicals and solvents were of either chromatographic or analytical grade.

### 2.2. Calibration and quality control solutions

Stock solutions containing 200 μg mL$^{-1}$ of ART and DHA were prepared in methanol. Working solutions (0.2, 0.4, 1, 2, 4, 20 and 40 μg mL$^{-1}$), used to provide the calibration curves, were obtained by appropriate dilution from the stock standards. The IS solution had a concentration of 20 μg mL$^{-1}$. All solutions were maintained in polypropylene tubes and stored protected from light at −20 °C.

Drug-free human plasma samples were obtained from blood donors and stored frozen at −20 °C. Prior to use, the plasma samples were allowed to thaw at room temperature.

Plasma quality control samples (QC) spiked with 15, 400 and 750 ng mL$^{-1}$ of ART and DHA were prepared and analyzed for validation purposes.

### 2.3. Liquid-phase microextraction procedure

Before extraction, the sample vial was filled with 1 mL plasma and diluted with deionized water to a total volume of 4 mL. The needles of two 1725RN gas tight syringes (250 μL, Hamilton, Reno, NV, USA) were used to connect the ends of a 7 cm piece of Accurel PP Q3/2 porous polypropylene hollow fiber (Membrana, Wuppertal, Germany), one serving to introduce the acceptor solution, while the second was used for collection of final extract (Fig. 2). The inner diameter of the hollow fiber used was 600 μm, the thickness of the wall was 200 μm, and the pore size was 0.2 μm. The membrane was first dipped into toluene:*n*-octanol (1:1, v/v) for 15 s to immobilize the solvent in the pores. After impregnation, 20 μL of the same organic phase was injected into the lumen of the hollow fiber with the inlet microlitre syringe and, subsequently, the membrane was placed in the sample solution. During the extraction, the samples were stirred at 1100 rpm using a 752A magnetic stirrer

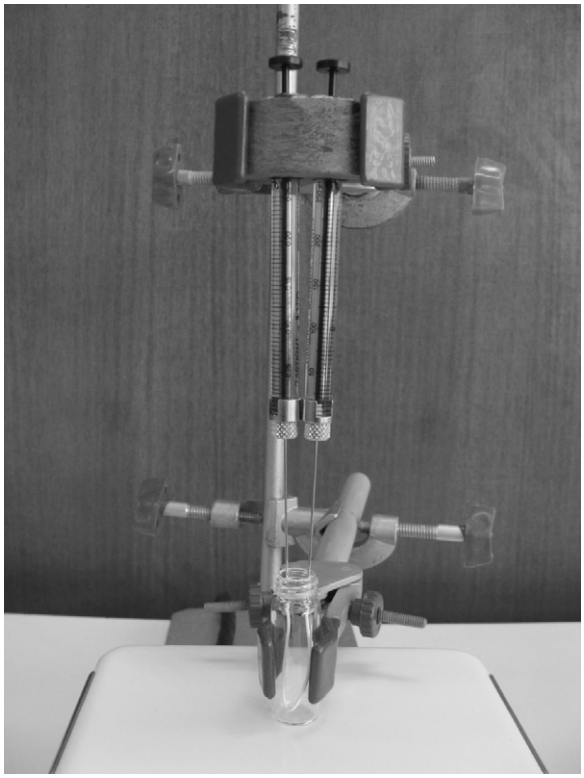

**Fig. 2.** Picture of the LPME apparatus used.

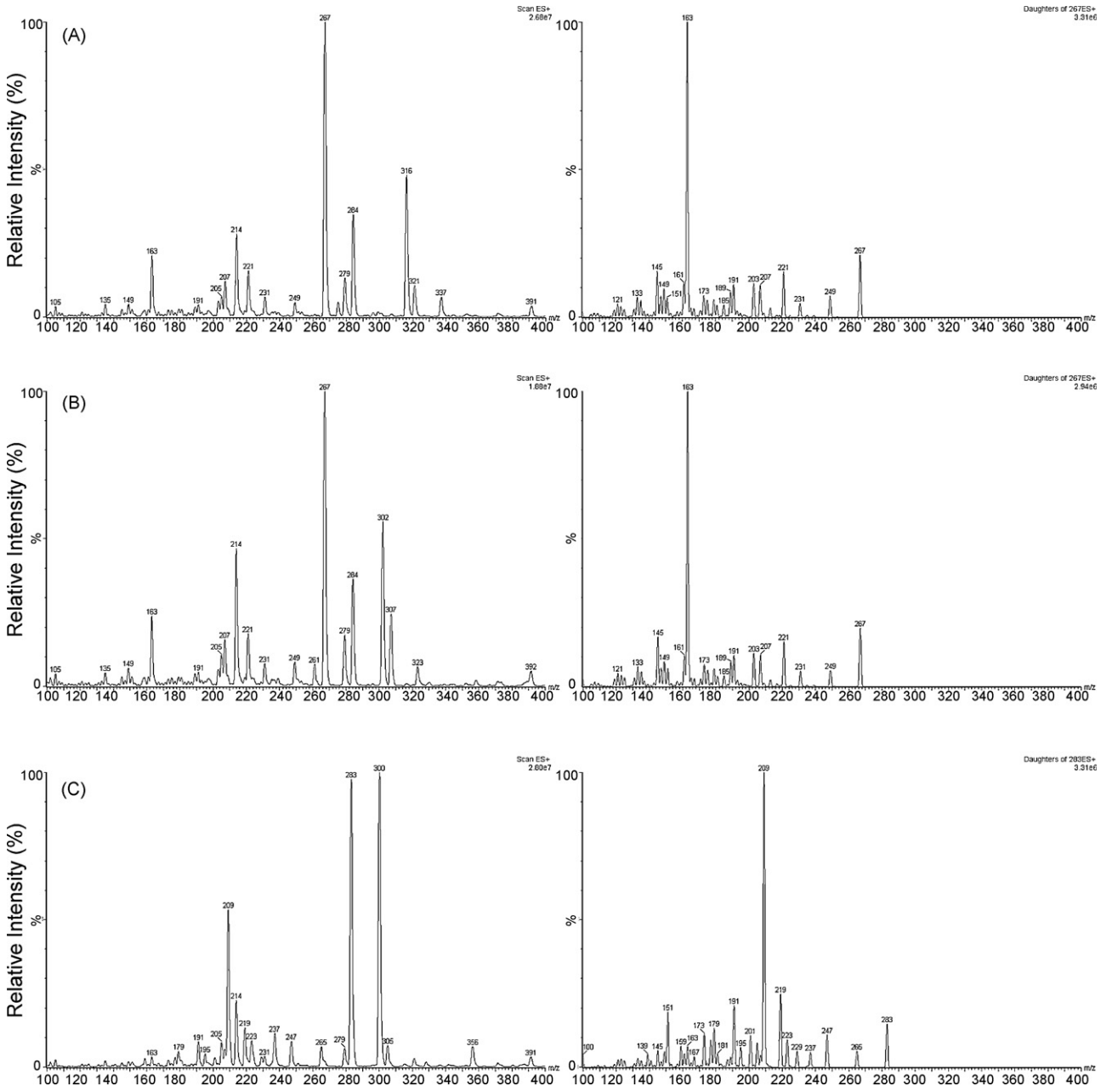


**Fig. 3.** Full scan mass spectra of the precursor ions (*left*) and ion products (*right*) of ART (A), DHA (B) and ATM (C) acquired during constant infusion of the analytes into the mass spectrometer.

(Fisatom, São Paulo, Brazil) with cylindrical-shaped stirring bars (10 mm × 3 mm) at room temperature (23 ± 2 °C). After extraction, the acceptor solution was withdrawn from the fiber by the outlet microlitre syringe and transferred into a conical polypropylene tube. The extracts obtained in the LPME procedure were evaporated to dryness under vacuum to prevent degradation of ART. The residues were then reconstituted in 100 μL of the LC mobile phase prior to the analysis and 50 μL of this solution were introduced into the LC system. In order to avoid carry-over effects, each hollow fiber membrane was used once.

### 2.4. Liquid chromatography–mass spectrometry

Analyses were conducted using a Shimadzu (Kyoto, Japan) liquid chromatograph, equipped with a LC-AT VP solvent pump unit, a CTO-AS column oven and a SCL-10ADvp controller. Injections were performed manually through a 50 μL loop with a Rheodyne model 7125 injector (Rheodyne, Cotati, CA, USA). A Valco tee connection was used to split the effluent from the column. A flow rate of approximately 0.1 mL min$^{-1}$ was directed into the inlet of a Quattro LC triple quadrupole mass spectrometer fitted with a Z-ESI interface (Micromass, Manchester, UK).

The resolution of the analytes was performed at 23 ± 2 °C on a laboratory-made column based on poly(methyltetradecylsiloxane) immobilized onto a doubly zirconized-silica support followed by endcapping—Si–Zr(PMTDS)ec (150 mm × 3.9 mm, 5 μm particle size) [24]. A C8 guard column (4 mm × 4 mm i.d., Merck) was used to protect the analytical column. The mobile phase used for the analysis consisted of methanol–ammonium acetate (10 mmol L$^{-1}$, pH 5.0, 80:20, v/v) at a flow rate of 1 mL min$^{-1}$. All mobile phases used were filtered and degassed prior to utilization.

MS–MS analysis was carried out in the positive ion mode. The temperatures of the source block and desolvation system were kept at 80 and 250 °C, respectively. Nitrogen was used as both drying and nebulizing gas and argon as the collision gas. MS parameters were optimized by direct infusion of the analytes and IS solutions prepared in the mobile phase at a flow rate of 20 μL min$^{-1}$. Quantitation was performed by multiple reaction monitoring (MRM) of the precursor ions and their corresponding product ions with a dwell time of 0.4 s (Fig. 3). Using the acquisition mode, the mass spectrometer was programmed to scan from 100 to 400 amu for each analyte. The mass spectra were obtained and the ion with higher intensity was selected (precursor ion). Later, each precursor ion was subjected to fragmentation. The acquisition mode was used again and scan was carried out in the range of 100–400 amu. Finally, the product ion with higher intensity in the acquired spectra was chosen. The transition $m/z$ 267 > 163 was monitored for ART and DHA and $m/z$ 283 > 209 for the IS. Conditions for each MS transition were tuned to attain higher sensitivity. Summarizing, collision energy and capillary voltage were set for all analytes at 7 eV and 2 kV, respectively. The cone voltages were maintained at 22 V for ART and DHA and 20 V for the IS. For this purpose, a MassLynx data sampling system (Micromass), version 3.5 was used.

### 2.5. Validation of the method

The absolute recovery of ART and DHA extracted from plasma samples ($n$ = 3) spiked with 15, 400 and 750 ng mL$^{-1}$ of the analytes was determined by comparing the areas obtained for these samples with the areas achieved by direct injection of pure solutions containing the same amount of each compound dissolved in the mobile phase. Recovery was expressed as percentage of the amount extracted.

Calibration curves having seven different concentrations were obtained by analyzing spiked plasma samples in triplicate over the concentration range of 5–1000 ng mL$^{-1}$ for ART and DHA. All samples also contained 500 ng mL$^{-1}$ IS. The results were plotted as a graph of peak area ratio *versus* analyte concentration and the best relationship was obtained by linear least-squares regression analysis. The linearity of the calibration curves was determined with the $F$-test for lack-of-fit ($F_{LOF}$) as described by Rosing et al. [25], using a $p$-value of 0.05. Statistical Product and Service Solutions (SPSS) for Windows, version 9.0.0 (SPSS Inc., Chicago, IL, USA), was used to perform all statistical calculations.

The sensitivity of the method was estimated by determining the limit of quantification (LOQ). The LOQ was defined as the lowest concentration of the analytes that could be determined with accuracy and precision below 20% over five analytical runs and was obtained from plasma samples spiked with 5 ng mL$^{-1}$ of ART and DHA.

To assess within-day precision and accuracy, five replicate analysis of plasma samples spiked at low, medium and high levels of ART and DHA were performed. For between-day assay, five determinations of each concentration level (as described for within-day assay) were analyzed over 3 days. Precision and accuracy were expressed as relative standard deviation (RSD, %) and relative error ($E$, %), respectively.

Freeze–thaw cycles and short-term room temperature stability assays were also evaluated. To perform the freeze–thaw cycle stability assay, three aliquots at low (15 ng mL$^{-1}$) and high (750 ng mL$^{-1}$) concentrations of the QC samples were stored at −20 °C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 12 h under the same conditions. This cycle was repeated once more. Finally, the samples were extracted and analyzed after the last cycle. For the determination of short-term room temperature stability assay, three aliquots of each QC sample (at the same concentrations as described above) were prepared and kept at room temperature (23 ± 2 °C) for 12 h. After this period, the samples were analyzed. The peak area ratio obtained from both stability assays were compared to the peak area ratio obtained with freshly prepared samples. Student's $t$-test was applied, using a $p$-value of 0.05 [25].

Matrix effects were assessed by comparing the peak areas obtained from the analysis of neat standard solutions dissolved in the mobile phase to the peak areas acquired from standard solutions spiked after extraction into extracts from five different sources of plasma as recommended by Matuszewski et al. [26]. The experiment was done in duplicate for the low (15 ng mL$^{-1}$) and high (750 ng mL$^{-1}$) concentrations of ART and DHA and for the concentration of the IS used in the developed method (500 ng mL$^{-1}$). The result was expressed as matrix effect (%) and RSD (%).

### 2.6. Application of the method

The method was further applied in a pilot study of kinetic disposition of the analytes in rats after the administration of ART. Young male Wistar rats weighing approximately 200 g had access *ad libitum* to feed and tap water and were acclimatized in a temperature- and humidity-controlled room under a 12 h light/dark cycle for one day before the beginning of the experiment.

After a 12 h fast, animals received by gavage a single oral dose (120 mg kg$^{-1}$) of ART dissolved in soybean oil. Following administration, blood samples were collected by decapitation at times of 0.5, 1, 2, 4, 6 and 8 h ($n$ = 3 per sampling time). Plasma was obtained by centrifugation at 2000 × $g$ for 10 min at 4 °C and stored at −20 °C until analyzed. The results obtained from these samples were used to yield pharmacokinetic profiles by plotting drug concentration *versus* time. The experimental protocol was approved by the Ethics Committee on the Use of Animals (CEUA) of the University of São Paulo at Ribeirão Preto (process number 06.1.907.53.4).

## 3. Results and discussion

### 3.1. Chromatographic resolution and mass detection

The separation of ART and DHA has traditionally been achieved using conventional cyano- [11–14] and octadecyl-bonded [15–18] stationary phases in the reversed-phase elution mode. Therefore, a promising alternative was employed for this purpose. The resolution of the analytes was attained on a laboratory-made stationary phase based on poly(methyltetrasdecylsiloxane) attached to a zirconized-silica support (Si–Zr–PMTDSec). This stationary phase was recently developed and demonstrated some desirable properties for pharmaceutical analysis, such as higher chemical and thermal stability along with the lower density of residual silanol groups compared with other phases [24]. No noticeable variation in the peak shapes or retention times of the analytes was observed during the development and application of the method, which confirmed the stability of the column using this mobile phase. Furthermore, the total run time of the separation obtained was below 5 min (Fig. 4), a very attractive characteristic in bioanalysis, especially when dealing with numerous samples (e.g., clinical trials).

Unlike the majority of compounds analyzed by MS, the protonated molecules of ART ($MH^+$) $m/z$ 299 and DHA ($MH^+$) $m/z$ 285 are not detected or present relative low intensity [18]. On the other hand, the fragments ($MH^+ - CH_3OH$) $m/z$ 267 and ($MH^+ - CH_3OH - C_2H_5OH$) $m/z$ 221 for ART and ($MH^+ - OH$) $m/z$ 267 and ($MH^+ - OH - C_2H_5OH$) $m/z$ 221 for DHA have frequently been monitored for quantification using ESI or APCI [15–17]. In the present study, both analytes had the same most abundant precursor ion ($m/z$ 267) and, after submitting these base peaks to collision-induced dissociation, the predominant product ion for

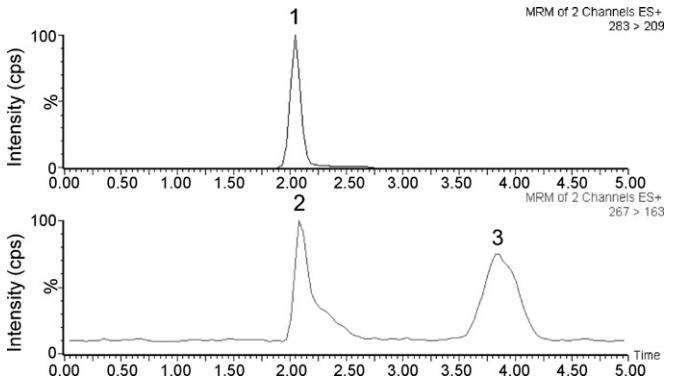


**Fig. 4.** MRM chromatograms referring to the analysis of ART and DHA after the two-phase LPME procedure in a plasma sample spiked with ART and DHA (10 ng mL$^{-1}$) and IS (500 ng mL$^{-1}$). Chromatographic conditions as in Section 2. Peaks: 1, IS; 2, DHA; 3, ART.

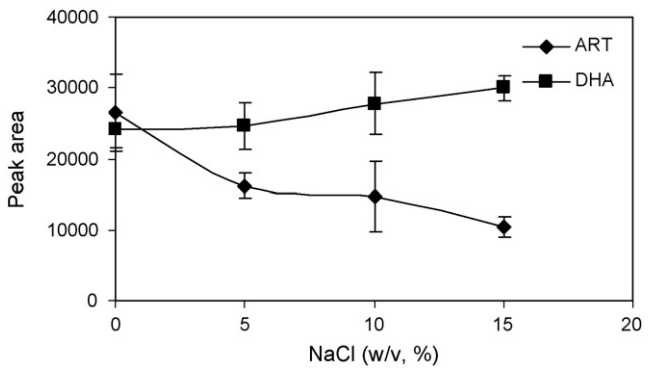


**Fig. 6.** Extraction efficiency (represented by peak areas) for ART and DHA with different amounts of salt added to the donor phase ($n$ = 3). Extraction conditions—organic phase: toluene–$n$-octanol (1:1); extraction time: 30 min; stir speed: 1100 rpm.

both analytes was again the same ion ($m/z$ 163) as shown in Fig. 3. This result was not a great problem for the development of the method since ART and DHA were chromatographically resolved prior to the MS detection and thereby a selective method regarding these analytes was still obtained. Conversely, the mass spectrum of the IS was completely different from the drug and metabolite and the ion transition $m/z$ 283 > 209 was effectively monitored for calculations.

### 3.2. Optimization of the LPME procedure

ATM and its derivatives are neutral molecules and, therefore, good candidates for two-phase LPME [27]. During the optimization of the LPME procedure for the extraction of ART and DHA, the selection of the organic phase, addition of salts to the donor phase and extraction time, some important variables in this mode of the LLE technique, were evaluated. The pH of the sample was not adjusted and simple dilution with water was performed to reduce the viscosity of the matrix as well as the interaction of the analytes with plasma proteins [28].

#### 3.2.1. Selection of the organic phase

In two-phase LPME, the organic solvent impregnated in the pores of the membrane also occupies the lumen of the hollow fiber and is considered to be of primary importance in the procedure since this parameter influences both the recovery and the selectivity of the method [29]. Hence, butyl ether, hexyl acetate, toluene and toluene:$n$-octanol (1:1, v/v) were studied as organic phases to extract the analytes from plasma. According to Fig. 5, although other organic phases provided the recovery of ART, only toluene–$n$-octanol (1:1, v/v) successfully removed DHA from the donor phase. As the metabolite (log $P$ = 2.6) is more polar than its lipophilic precursor (log $P$ = 3.0), this compound may require solvents with higher polarity for efficient solvation (e.g., $n$-octanol). An experiment was also performed to evaluate pure $n$-octanol as organic phase but negligible extraction was attained (data not shown). Despite the fact that a mobile phase with high content of organic solvent was used, the direct injection of extracts into the chromatographic system was not possible due to the deformation of peak shapes along with the modification of retention times of the analytes. For this reason, the extracts were dried and the residues were dissolved in the mobile phase resuspended before analyses.

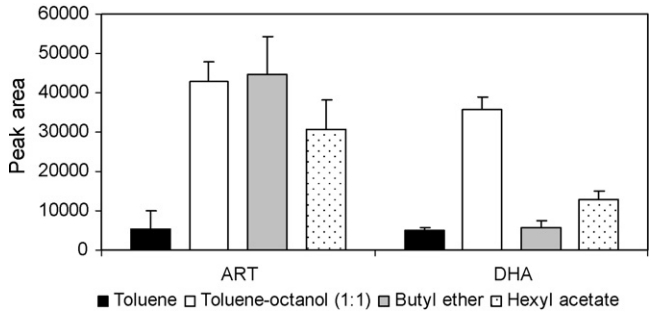


**Fig. 5.** Extraction efficiency (represented by peak areas) for ART and DHA with different organic phases ($n$ = 3). Extraction conditions—extraction time: 30 min and stir speed: 1100 rpm.

#### 3.2.2. Addition of salts to the donor phase

The approach of adding reasonable amounts of salts to the sample to obtain the salting-out effect is often carried out in LLE procedures and, more recently, applied to microextraction techniques [30]. Some methods described in the literature for the determination of ART and DHA in plasma also employed this strategy [11,13,15,16]. Thus, the addition of salts to the donor phase was conducted using different quantities of sodium chloride (5, 10 and 15%, w/v). As observed in Fig. 6, distinct results were found for the drug and its metabolite since a sligthly higher extraction efficiency was obtained for DHA with addition of salts whereas the extraction of ART was notably decreased. The high viscosity of the plasma sample and/or modification of the Nerst diffusion layer prompted by the addition of salts may have predominantly disturbed the diffusion of the parent drug towards the organic phase [31]. On the other hand, the salting-out effect may have balanced this process for the hydrophilic metabolite and higher recoveries were observed for this analyte when using salts.

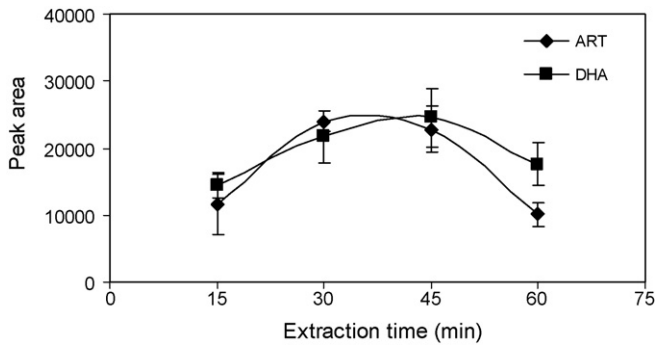


**Fig. 7.** Extraction efficiency (represented by peak areas) for ART and DHA with different extraction times ($n$ = 3). Extraction conditions—organic phase: toluene–$n$-octanol (1:1) and stir speed: 1100 rpm.

**Table 1**
Mean absolute recoveries and limit of quantification of the method for the analysis of ART and DHA in plasma samples.

| Analyte | Recovery (%) | RSD (%) | Observed concentration[a] (ng mL$^{-1}$) | RSD (%) | $E$ (%) |
|---|---|---|---|---|---|
| ART | 32.5 | 8.0 | 4.9 | 6.0 | −2.0 |
| DHA | 25.8 | 7.4 | 5.8 | 13.3 | 16.0 |

[a] Initial concentration, 5 ng mL$^{-1}$; RSD, relative standard deviation; $E$, relative error.

**Table 2**
Linearity of the method for the analysis of the ART and DHA in plasma samples.

| Analyte | Range (ng mL$^{-1}$) | Linear equation | $r^2$ | ANOVA lack-of-fit | |
|---|---|---|---|---|---|
| | | | | $F$-value | $p$-Value |
| ART | 5–1000 | $y = 0.035x - 0.0323$ | 0.9931 | 0.02 | 0.99 |
| DHA | 5–1000 | $y = 0.002x + 0.0152$ | 0.9925 | 1.47 | 0.30 |

$r^2$, coefficient of determination.

### 3.2.3. Extraction time

LPME is based on the equilibrium between the analyte in the sample and in the acceptor phase supported on the hollow fiber membrane and, consequently, the evaluation of different extraction times in this procedure is essential to reveal the kinetics of the process [32]. The extraction-time profile was established over the range of 15–60 min of exposure (Fig. 7). In this experiment, the extraction of ART and DHA quickly increased up to about 30 min and then gradually decreased in the subsequent time periods. The slow evaporation or even the dissolution of the organic phase into the sample after prolonged periods may have contributed to this result. Indeed, two-phase LPME procedures are typically performed at short times to prevent such problems [33]. Therefore, the extraction time was set at 30 min for method validation. As the procedure does not require any specialized apparatus and owing to the low cost of each extraction unit, several samples may be processed at the same time.

## 3.3. Validation of the method

The validation of the method was carried out according to the recommendations described in the literature [34]. Some important parameters, such as linearity, recovery, precision, accuracy, limit of quantification, stability and matrix effects assays were evaluated. Internal standardization was adopted for quantification and thereby ATM, which presents structural similarities to ART and DHA, was selected and added to samples at the concentration of 500 ng mL$^{-1}$. The IS had chromatographic performance as well as extraction profile (~35%) quite similar to the analytes studied.

**Table 4**
Stability test of the method for the analysis of ART and DHA in plasma samples.

| Analyte | $p$-Value | | | |
|---|---|---|---|---|
| | Freeze–thaw cycles | | Short-term room temperature | |
| | Low[a] | High[b] | Low[a] | High[b] |
| ART | 0.20 | 0.63 | 0.26 | 0.74 |
| DHA | 0.12 | 0.19 | 0.00 | 0.00 |

[a] Low concentration (15 ng mL$^{-1}$).
[b] High concentration (750 ng mL$^{-1}$).

**Table 5**
Matrix effect test of the method for the analysis of ART and DHA in plasma samples.

| Analyte | Matrix effect (%) | RSD (%) |
|---|---|---|
| 15 ng mL$^{-1}$ | | |
| ART | 91.0 | 4.4 |
| DHA | 95.1 | 3.6 |
| 750 ng mL$^{-1}$ | | |
| ART | 87.8 | 9.1 |
| DHA | 99.6 | 2.6 |
| 500 ng mL$^{-1}$ | | |
| ATM (IS) | 99.7 | 10.2 |

The mean absolute recoveries using the optimized LPME procedure were 32 and 25% for ART and DHA, respectively, with RSD values lower than 15% for both analytes (Table 1). The higher rates observed for ART probably resulted from the greater lipophilicity of the drug, which implies higher affinity for the organic phase. Even though the values achieved herein were lower than those reported in other methods for these analytes, comparable sensitivity was still obtained (LOQ = 5 ng mL$^{-1}$; Table 1). Additionally, the recoveries are consistent with other results observed with several other drugs employing LPME in plasma samples [35].

The method was linear over the concentration range of 5–1000 ng mL$^{-1}$ using linear least-square regression with suitable coefficients of determination of 0.9931 and 0.9925 for ART and DHA, respectively. The linearity of the method was also confirmed by the lack-of-fit test (Table 2). The RSD and $E$ values achieved for the three concentrations evaluated in within- and between-day assays are shown in Table 3. These values were <15% and are in good agreement with the recommendations found in the recent literature [34].

According to Table 4, ART and DHA spiked in plasma samples were stable when submitted to freeze and thaw cycles ($p$-values > 0.05). On the other hand, DHA showed considerable

**Table 3**
Precision and accuracy of the method for the analysis of ART and DHA in plasma samples.

| | Within-day ($n$ = 5)[a] | | | Between-day ($n$ = 3)[b] | | |
|---|---|---|---|---|---|---|
| | Concentration (ng mL$^{-1}$) | RSD (%) | $E$ (%) | Concentration (ng mL$^{-1}$) | RSD (%) | $E$ (%) |
| 15 ng mL$^{-1}$ | | | | | | |
| ART | 15.0 | 5.2 | 0.0 | 15.7 | 8.7 | 4.6 |
| DHA | 15.3 | 5.1 | 2.0 | 14.9 | 7.4 | −0.6 |
| 400 ng mL$^{-1}$ | | | | | | |
| ART | 428.5 | 3.8 | 7.1 | 428.8 | 4.2 | 7.2 |
| DHA | 373.2 | 4.4 | −6.7 | 376.2 | 6.2 | −5.9 |
| 750 ng mL$^{-1}$ | | | | | | |
| ART | 772.1 | 3.6 | 2.9 | 751.5 | 5.1 | 0.2 |
| DHA | 692.8 | 6.2 | −7.6 | 675.9 | 6.6 | −9.8 |

RSD, relative standard deviation; $E$, relative error.
[a] Number of samples.
[b] Number of days.

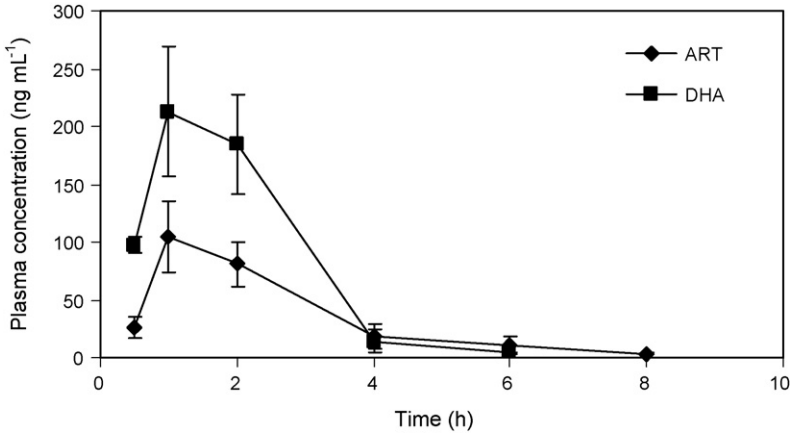


**Fig. 8.** Mean (±SD) plasma concentrations *versus* time curves of ART and DHA in rats following a single oral dose of the drug (120 mg kg$^{-1}$; $n = 3$ per sampling time).

degradation when samples were maintained for 12 h at room temperature ($p$-values < 0.05). The poor stability of the metabolite in plasma when kept prolonged periods at room temperature has already been described in some reports [17,18]. Therefore, all samples were processed within 2 h to avoid losses of DHA, as suggested by Shi et al. [17]. The ion suppression as well as its variability employing the LPME procedure was below 15% for all analytes in the concentrations studied, which indicates acceptable matrix effects for the method (Table 5).

### 3.4. Application of the method

The plasmatic disposition of the drug and its metabolite in rats receiving a single oral dose of ART is shown in Fig. 8. Both analytes were rapidly eliminated from plasma and the concentrations of DHA were considerably higher than those of ART, suggesting an extensive pre-systemic metabolism of the drug [5]. In addition, these results corroborate a study previously published regarding the pharmacokinetics of ART and DHA in rats [36].

## 4. Conclusions

A method for the determination of ART and DHA in plasma employing two-phase LPME for sample clean-up prior to LC–MS–MS was developed. The LPME procedure described in this article is affordable and solvent consumption during sample preparation was greatly reduced compared with other methods reported for these analytes.

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