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Development and validation of a high-performance liquid chromatography–mass spectroscopy assay for determination of artesunate and dihydroartemisinin in human plasma

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Abstract

A sensitive method has been developed and validated for the determination of artesunate and its active metabolite dihydroartemisinin (DHA) in human plasma using artemisinin as an internal standard. Solid phase extraction (SPE) using Oasis HLB extraction cartridges was used for sample preparation and analysis was performed on a Shimadzu LCMS-2010 in single ion monitoring positive mode using atmospheric pressure chemical ionization (APCI) as an interface. Positive ions were measured using extracted ion chromatogram mode. The extracted ion for artesunate, α- and β-DHA was *m*/*z* 221 and for artemisinin was *m*/*z* 283. Chromatography was carried out using a Synergi Max-RP, 4 μ, 75 mm × 4.6 mm column using glacial acetic acid 0.1%, acetonitrile and methanol mixture (38:46.5:15.5) as a mobile phase delivered at a flow rate of 0.5 mL/min. The retention times of artesunate, α- and β-DHA and artemisinin were 17.4, 11.8, 18.7 and 13.4 min, respectively, with a total run time of 21 min. The assay was linear over the range 1–3000 ng/mL for artesunate and DHA. The analysis of quality control samples for artesunate 50, 300, 1300 and 2600 ng/mL demonstrated excellent precision with relative standard deviation of 14.3, 11.3, 7.5 and 12.1%, respectively ($n = 5$). Recoveries at concentration of 50, 300, 1300 and 2600 ng/mL were 75, 94.5, 74.3 and 75.5%, respectively; similar results were obtained for precision and recovery of DHA. This liquid chromatography–mass spectroscopy (LC–MS) method for the determination of artesunate and DHA in human plasma has superior specification for sensitivity, sample throughput and robustness than previous methods and can reliably quantitate concentrations of both (artesunate and DHA) compounds as low as 1 ng/mL.

Keywords: Artesunate; Antiparasitic; Liquid chromatography–mass spectroscopy; Dihydroartemisinin; Antimalarial

1. Introduction

Malaria is a life threatening parasitic diseases transmitted by Anopheles mosquitoes. It is a common and serious tropical disease which continues to be a major public health problem world-wide causing disease and death throughout most of the developing world. It has been reported by WHO in 1997 that malaria causes 1.5 million to 2.7 million deaths each year, with an estimated 300–500 million cases annually [1].

Artemisinin and its derivatives such as artesunate (ARTS) and artemether have become an integral component of malaria treatment protocols in Asian and African countries for over 15 years especially against chloroquine resistant falciparum malaria [2]. Artemisinin is a naturally occurring sesquiterpene lactone containing an endoperoxide group. This drug was first extracted from a Chinese herb *Artemisia annua* (QINGHAO). The free acid of ARTS is a lipophilic substance, very slightly soluble in water [3]. Structures of ARTS, α- and β-dihydroartemisinin (DHA) are shown in Fig. 1.

ARTS is a hemisuccinate ester of DHA and is highly effective in the treatment of malaria. It is clinically useful in the treatment of acute malaria and is particularly effective against chloroquine resistant strains of falciparum malaria. Similar to other derivatives of artemisinin, ARTS is rapidly

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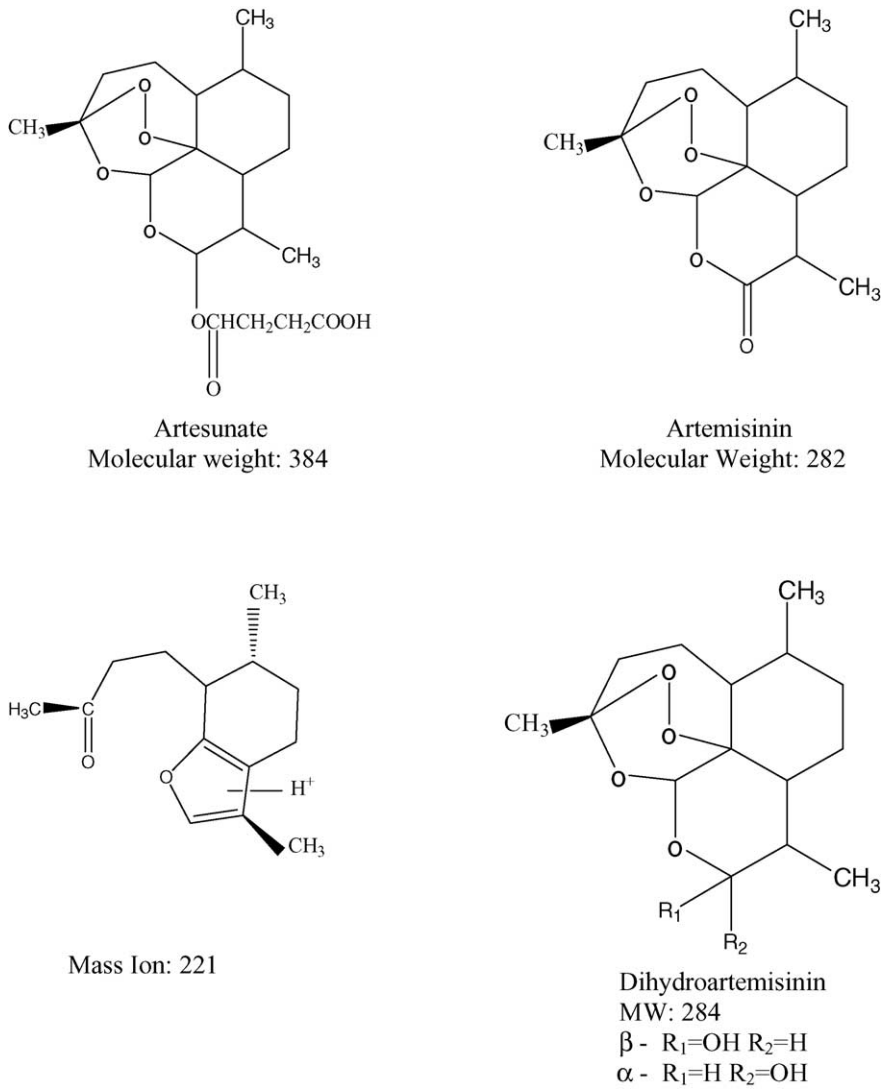

Fig. 1. Chemical structures of artesunate (top left), internal standard artemisinin (top right), dihydroartemisinin (bottom right) and mass fragment (bottom left).

converted to its active metabolite DHA in vivo which is responsible for the antimalarial action. Artemisinin and its derivatives are nitrogen-free sequiterpenes containing a peroxide linkage which confers activity against malarial parasites. The endoperoxide linkage causes free radical damage to parasitic membrane systems, leading to death of the organism. Recently, artemisinin was shown to inhibit *P. falciparum* PfATP6, a calcium dependent ATPase [4].

Other reported analytical methods for ARTS were unsuited for clinical pharmacokinetic studies because they are unable to measure all compounds simultaneously with good separation, lack sensitivity or have plasma interferences. Since artemisinin and its derivatives do not have ultraviolet or fluorescent chromophores, analysis of these drugs in biological fluids has proven difficult. The pharmacokinetics of ARTS and its active metabolite DHA have been previously characterized by high-performance liquid chromatography (HPLC) using post column alkali decomposition [5] and electrochemical detection [6,7]. However, ECD methods are difficult to use routinely as these require equipment maintained with a constant oxygen free environment, have sensitivity limited to 5–10 ng/mL and are labor-intensive. Liquid chromatography–mass spectroscopy (LC–MS) gives definitive identification and the quantitative determination of compounds, provides high sensitivity and selectivity for all analytes of interest.

We describe a specific and sensitive method, which uses an internal standard, for the determination of ARTS and its metabolite DHA in human plasma for anticipated clinical trials with this antimalarial agent.

2. Experimental

2.1. Solvents and chemicals

ARTS (Lot # 20001215), DHA (Lot # 20010521) and artemisinin were obtained from Shin Poong Pharm. Co. Ltd

(Yoksam-Dong, Seoul, Korea). DHA is a mixture of α and β tautomers of unknown ratio. Acetonitrile, methanol, ethyl acetate, butyl chloride (HPLC Grade) was obtained from Fischer Scientific (Fair Lawn, NJ, USA). Analytical grade glacial acetic acid was obtained from Mallinckrodt Baker Inc (Paris, KU, USA). Ultra-pure analytical grade Type 1 water for HPLC was produced by Milli-Q Plus water system (Millipore Corporation, Bedford, MA, USA) and used for the preparation of the sample and aqueous solutions.

2.2. Standards

Standard stock solution of ARTS, DHA and artemisinin solution were prepared in methanol. ARTS, DHA and artemisinin were weighed on a Mettler Toledo AG 104 analytical balance (Mettler Toledo, Inc., Hightstown, NJ, USA). Appropriate amounts of drugs were dissolved using methanol in volumetric flasks to make a 1 mg/mL stock solution of each. For calibration curves concentrations of 1, 25, 100, 200, 400, 600, 1200, 1800, 2400 and 3000 ng/mL were prepared by serial dilution of a stock solution (1 mg/mL in methanol) with methanol. A 100 μg/mL solution for internal standard was also prepared in methanol. The stock solution was stored at −80 °C as compound is unstable at room temperature when exposed to light [8].

2.3. Instrumentation

Chromatographic analysis was carried out on a Shimadzu Model 2010 liquid chromatograph and mass spectrometer (Shimadzu, Columbia, MD, USA) using LC-10AD Solvent Delivery system (Pump: A, B). The injection is made with a Shimadzu SIL-10AD automatic injector and analysis uses Shimadzu model 2010 data analysis software Lab Solutions Version 3. Samples were stored in a ULT 2586-5-A14 freezer (Revco scientific, Asheville NC, USA) at −80 °C.

2.4. Sample preparation

This procedure was validated by spiking human plasma with known concentrations of ARTS, DHA followed by internal standard. Outdated human plasma was obtained from the blood bank of University of Iowa Hospitals and Clinics and stored frozen in aliquots at −80 °C. Extraction of ARTS, DHA and artemisinin was carried out by solid phase extraction (SPE). OASIS HLB® 1 cc (30 mg) SPE cartridges (Waters) were used for the solid phase extraction. Plasma aliquots (0.5 mL) were spiked with 2.4 μg of artemisinin (12 μL of 100 μg/mL solution) as internal standard. The cartridges were initially primed with 1 mL of methanol followed by 1 mL of 1 M acetic acid. Plasma sample was applied under reduced vacuum (i.e. 4 mmHg). After aspiration, each cartridge was washed with 2 mL of 1 M acetic acid followed by 1 mL of 20% methanol in 1 M acetic acid at reduced vacuum (5 mmHg). The analytes were eluted using 2 mL of 40% ethyl acetate in butyl chloride under reduced vacuum (5 mmHg). The non aqueous step was applied immediately after the washing step. The eluent was evaporated by stream of nitrogen. The residue was reconstituted with 200 μL of mobile phase and 25 μL was injected for lower calibration levels (1–600 ng/mL) and 5 μL (600–3000 ng/mL) was injected for higher calibration levels.

2.5. Chromatographic and mass spectrometer conditions

The mobile phase used for the analysis consisted of acetic acid (0.1%):methanol:acetonitrile (38:46.5:15.5, v/v). The mobile phase was filtered before being used to prevent entry of bubbles or impurities in the system. The solution was degassed and sonicated completely with vacuum for approximately 15 min before use. The mobile phase was delivered at a flow rate of 0.5 mL/min. The analysis was carried out using Synergi Max-RP 80A HPLC column, 75 mm × 4.6 mm, 4 μ (Phenomenex, USA) using a guard column(Phenomenex, USA) with C-12 max-RP cartridges.

APCI, curved desolvation line (CDL), heat block temperatures for the analysis were set at 400, 200 and 200 °C, respectively. Temperatures were varied but these range of values resulted in highest peak area and sharp peak shape. The nebulizer gas flow was set at 2.5 L/min; the detector voltage was set at 1.6 EV, and ion source polarity was set in positive mode. The flow was directed to mass spectrometer 7 min after the start of the run.

2.6. Validation

2.6.1. Selectivity

Six human plasma samples with six individual donors receiving no medication were extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent response at the retention time of ARTS, DHA and artemisinin was compared to the lower limit of quantification.

2.6.2. Accuracy and precision

To validate the method for ARTS and DHA, intra-day accuracy and precision were evaluated by analysis at various concentration levels on the same day. Four different concentrations (50, 300, 1300, 2600 ng/mL) levels were selected to cover the entire range of calibration curve. Analysis was being done by preparing five samples at each concentration level and concentrations were calculated from calibration curves. The upper limit of quantification (ULOQ) was given by highest level of the calibration curve. To assess the inter-day accuracy and precision, the intra-day assay was repeated on three different days.

2.6.3. Recovery

For the recovery experiment, recovery samples were made by spiking both ARTS and DHA at the four different concentration levels, but without artemisinin prior to extraction. After evaporation of sample solvent, artemisinin was

added. For recovery controls, blank plasma was extracted. After evaporation of sample solvent, the appropriate amount of ARTS, DHA and artemisinin were added. The analysis was done in pentuplate at each concentration levels. Recovery of artemisinin was carried out by comparing the area obtained from an extracted sample and a standard.

2.6.4. Freeze/thaw analysis

Analysis in pentuplate was performed on 50, 300, 1300 and 2600 ng/mL quality control (QC) samples. QC samples were stored at −80 °C for 24 h. Aliquots were thawed unassisted at room temperature. When completely thawed, the samples were refrozen for approximately 24 h at −80 °C. These freeze–thaw samples were analyzed along with QC's to see if there is any variation due to thawing of the samples. The stability data was used to support request for repeat analysis.

2.6.5. Calibration and sample quantification

Two calibration curves were prepared in the concentration range of 1–3000 ng/mL of the plasma ($n = 1$, at each level) for ARTS and DHA. The lower calibration curve extended from 1 to 600 ng/mL with standards concentration at 1, 25, 100, 200, 400, and 600 ng/mL, and the higher calibration curve extended from 600 to 3000 ng/mL with standards at 600, 1200, 1800, 2400, and 3000 ng/mL. The analysis was done using artemisinin as an internal standard and ratio of parent compound to internal standard was plotted against concentration per milliliter of plasma. The calibration curve was linearly fitted and weighed by 1/concentration. Concentrations of compounds in samples were calculated from the calibration curve of ARTS and DHA.

2.6.6. Stability

2.6.6.1. Storage stability. The stability of ARTS and its metabolite in human plasma was investigated over a period of 2 months. Spiked samples were prepared with drug free plasma at four different concentrations: 50, 300, 1300, 2600 ng/mL ($n = 5$). Spiked human plasma samples were stored frozen at −80 °C. Aliquots at each level were thawed and analyzed at 0, 40 and 60 days. A standard calibration curve was freshly prepared on the day of analysis, and concentration levels are measured on the basis of calibration curve.

2.6.6.2. Bench stability. Samples were prepared at various QC levels (50, 300, 1300 and 2600 ng/mL) and kept on working bench for 24 and 48 h before analysis. These studies were carried out to predict the extent of degradation when samples are kept on bench for more than 24 h. Analysis was done in pentuplate at each concentration levels.

2.6.6.3. Autoinjector stability. Stability of samples in autoinjector was carried out for over 30 h by injecting the same extracted plasma sample, spiked with 600 ng/mL of ARTS, DHA and 2.4 μg/mL of artemisinin, at intervals of 3 h. The stability was carried over a period of 30 h.

3. Results

3.1. Mass spectral analysis

When ARTS and DHA were injected directly in the mass spectrometer along with the mobile phase with a positive ion interface, then protonated molecules $(MH)^+$ of ARTS and DHA were not seen in abundance, but a fragment of the parent and metabolite molecule was observed having a mass of *m*/*z* 221 both for ARTS and DHA while for internal standard we were able to detect the parent mass of *m*/*z* 283 [8]. The analysis temperature, nebulizer gas, and APCI temperature were selected to optimize specificity and sensitivity of *m*/*z* 221 and *m*/*z* 283 ion detection. The full scan mass spectra for ARTS, DHA and artemisinin are shown in Fig. 2A, B and C, respectively

3.2. Separation and relative retention time

Observed retention times were 12.1, 17.5, 18.5, and 13.4 min for α-DHA, ARTS, β-DHA and artemisinin, respectively, with a total run time of 21 min. The α and β tautomers of DHA were separated, however only the α tautomer of DHA was taken into account for quantification. Under the chromatographic condition, the ratio of α and β remain constant around 3.8 (α/β). Blank plasma was tested for endogenous interference. No additional peaks due to endogenous substances were observed that would interfere with the detection of compounds of interest. Typical chromatograms are shown in Fig. 3. A representative chromatogram of the plasma blank is shown in Fig. 3A. Fig. 3B shows a chromatogram calibration standard containing 200 ng/mL of ARTS and α and β-DHA. Fig. 3C shows a chromatogram calibration standard containing 2.4 μg/mL of artemisinin. Compared to other reported analytical methods, the LC–MS procedure results in much cleaner chromatograms [5–7].

3.3. Linearity

Calibration curve parameters for ARTS and DHA are shown in Table 1. Results were calculated using peak area

Table 1
Assay Linearity of method ($n = 5$)

Drug	Slope	*y*-intercept	Coefficient of correlation
Artesunate			
Lower calibration mean	0.003318	−0.00088	0.9959
Higher calibration mean	0.003228	−0.05355	0.9954
Dihydroartemisinin			
Lower calibration mean	0.002835	−0.04691	0.9973
Higher calibration mean	0.002708	−0.25844	0.9964

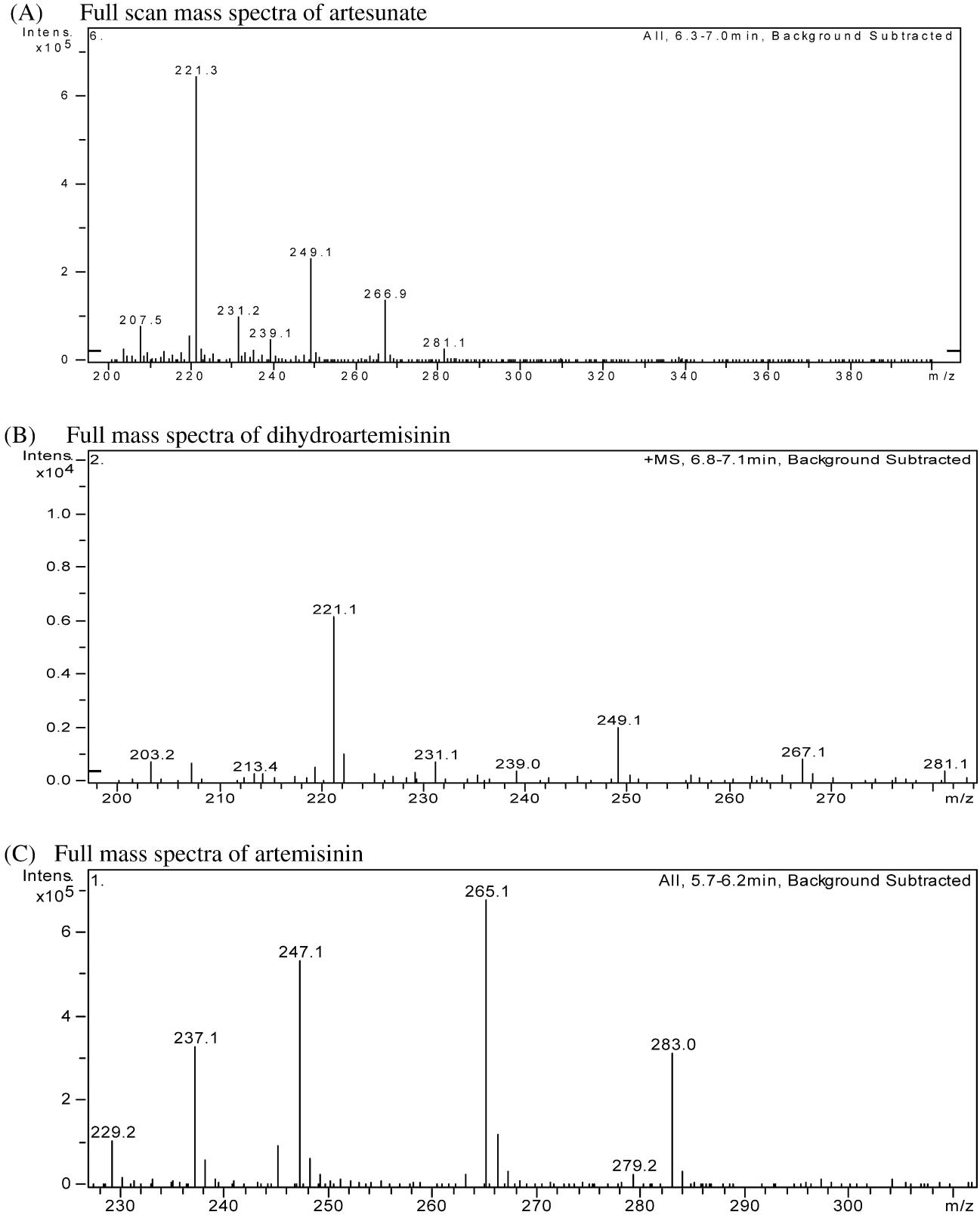

Fig. 2. Full mass spectra scan for ARTS (A) dihydroartemisinin (B) and internal standard artemisinin (C).

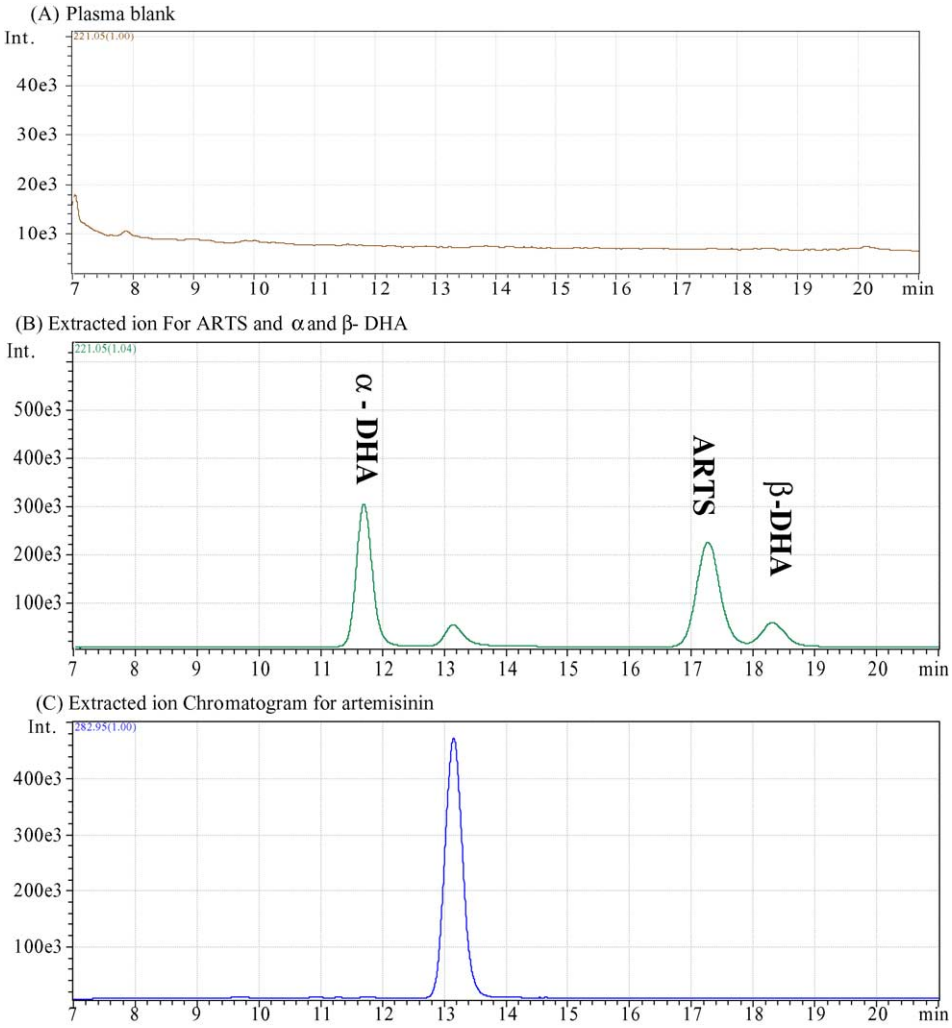

Fig. 3. Chromatograms of 25 μL injection blank plasma (A), calibration standard containing 200 ng/mL artesunate and dihydroartemisinin in human plasma (B) and internal standard artemisinin 2.4 μg/mL in human plasma (C).

ratios. Calibration curves for ARTS and DHA were linear using weighted (1/concentration) linear regression in the concentration range of 1–3000 ng/mL on all 5 days, with a correlation coefficient greater than or equal to 0.993 for all curves. The calibration curves accuracy is presented in Table 2, demonstrating that measured concentration is within 15% of the actual concentration. The limit of quantification has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 15% ($n = 5$) and signal to noise ratio of 5:1 for a pentuplate analysis both for ARTS and DHA. Results at lowest concentration studies (1 ng/mL) met the criteria for limit of detection. Linear calibration curves were obtained with a coefficient of correlation usually higher than 0.993 both for ARTS and DHA. Two calibration curves concentration ranges were selected because a single calibration curve covering the entire concentration range from 1 to 3000 ng/mL showed nonlinearity at higher concentration.

Table 2
Back calculated concentration from calibration curves ($n = 5$)

Nominal concentration (ng/mL)	Artesunate accuracy (%)	Dihydroartemisinin accuracy (%)
1	113	106
25	90	89
100	92	97
200	104	106
400	102	99
600	98	102
1200	101	95
1800	101	95
2400	100	101
3000	95	97

Accuracy: 100% measured concentration/nominal concentration.

3.4. Recovery data

Percentage recovery of ARTS and DHA was measured by dividing the ratio of concentration levels with that of con-

Table 3
Intra-day and inter-day precision and accuracy for ARTS in human plasma

	Theoretical concentration (ng/mL)			
	50	300	1300	2600
Intra-day run				
Overall mean ($n = 5$)	47.4	311.3	1282	2422
S.D.	6.8	35.3	95.9	294
CV (%)	14.3	11.3	7.5	12.1
DMT (%)	−5.2	3.6	−1.4	−6.9
Inter-day run				
Overall mean ($n = 15$)	45.7	273	1251	2678
S.D.	5.9	39.1	137	371
CV (%)	13	14.3	11	14.9
DMT (%)	−8.6	−9	−10.8	−4.3

S.D., standard deviation; CV, coefficient of variation; DMT, deviation of mean value from nominal.

Table 4
Intra-day and inter-day precision and accuracy for DHA in human plasma

	Theoretical concentration (ng/mL)			
	50	300	1300	2600
Intra-day run				
Overall mean ($n = 5$)	51.1	343	1307	2705
S.D.	7.4	32.7	115	364
CV (%)	14.5	9.5	8.8	13.5
DMT (%)	2.2	14.3	0.53	4.03
Inter-day run				
Overall mean ($n = 15$)	49.6	300.1	1251	2719
S.D.	6.95	45.8	149	371
CV (%)	14	15	11	13.7
DMT (%)	−0.9	0.0	−3.7	4.5

[a]S.D., standard deviation; CV, coefficient of variation; DMT, deviation of mean value from nominal.

trols. The mean recoveries ($n = 5$) for ARTS (50, 300, 1300, 2600 ng/mL) were 75%, 94.5%, 74.3% and 75.5%; DHA (50, 300, 1300, 2600 ng/mL) were 66.5%, 87.5%, 75% and 75.8%, respectively. Mean recovery ($n = 5$) for internal standard was 100 ± 12%

3.5. Accuracy and precision

The intra-day coefficients of variation for ARTS samples (50, 300, 1300, 2600 ng/mL) were 14.3%, 11.3%, 7.5% and 12.1%, and for DHA samples (50, 300, 1300, 2600 ng/mL) the results were 14.5%, 9.5%, 8.8% and 13.5%, respectively. Coefficients of variation of inter-day analysis of ARTS samples (50, 300, 1300, 2600 ng/mL) were 13%, 14.3%, 11% and 14.9%, and for DHA (50, 300, 1300, 2600 ng/mL) were 14%, 15%, 11.2% and 13.7%, respectively. The data obtained both for the ARTS and DHA was within the acceptable limits to meet guidelines for bioanalytical methods guidelines for bioanalytical validation [9]. Data for accuracy and precision are shown in Tables 3 and 4.

3.6. Storage stability data at −80°C

The sample long term stability at −80 °C was evaluated to establish acceptable storage conditions for clinical samples. Spiked human samples with known concentration of ARTS and DHA were analyzed at regular intervals. A standard calibration curve was freshly prepared on the day of analysis and actual concentration was obtained based on the calibration curve. The coefficient of variation for ARTS and DHA ranged from 4.9 to 15 and 3.6 to 12.2, respectively. Percentage recovery for ARTS and DHA varied from 100 ± 20 and 100 ± 16, respectively. Thus the result indicated that the drug was stable at −80 °C for at least 60 days. Stability data are shown in Tables 5 and 6.

3.7. Autoinjector stability

Stability of samples stored in the autoinjector was carried out over a period of 30 h by injecting same sample at an interval of 3 h. The CV for the peak area ratio of ARTS and DHA measurements were 8.3 and 4.9%, respectively. Concentration of ARTS and DHA varied from 667.8 to 602.8 and 690.4 to 663.8 ng/mL, respectively. These results demonstrate that ARTS and DHA samples are stable up to 30 h in the autoinjector. Results of autoinjector stability are shown in Table 7.

3.8. Freeze/thaw analysis

The freeze–thaw stability of ARTS and DHA was determined measuring the accuracy and precision for samples that underwent three freeze–thaw cycles. The results showed that ARTS and DHA were stable in human plasma through three freeze–thaw cycles. The precision ranged from 2.8 to 13.2% and the accuracy ranged from 99 to 116% for ARTS. The

Table 5
Stability data for ARTS at −80 °C

Spiked concentration (ng/mL)	Days								
	0			40			60		
	Mean concentration (ng/mL)	CV (%)	Recovery (%)	Mean concentration (ng/mL)	CV (%)	Recovery (%)	Mean concentration (ng/mL)	CV (%)	Recovery (%)
50 ($n = 5$)	47	6.8	94	49	6.5	98	60	15	120
300 ($n = 5$)	324	9.3	108	321	9.1	107	321	11	107
1300 ($n = 5$)	1192	4.9	91.7	1126	7.7	86.7	1034	14	79.5

n, number of replicates.

Table 6
Stability data for DHA at −80 °C

Spiked concentration (ng/mL)	Days								
	0			40			60		
	Mean concentration (ng/mL)	CV (%)	Recovery (%)	Mean concentration (ng/mL)	CV (%)	Recovery (%)	Mean concentration (ng/mL)	CV (%)	Recovery (%)
50 (n = 5)	58	6.4	116	51.6	6.7	103.2	46	12.2	92
300 (n = 5)	320	9.8	106	302	9.6	100.6	285	10	95
1300 (n = 5)	1294	3.6	99.5	1176	6.3	90.5	1292	8.7	99.4

n, number of replicates.

Table 7
Autoinjector stability over 30 h for ARTS and DHA

Drug	Autoinjector stability mean ratio of drug/IS over 30 h	CV (%)
ARTS (n = 10)	0.86 ± 0.06	8.3
DHA (n = 10)	0.99 ± 0.04	4.9

precision ranged from 2.5 to 15% and accuracy ranged from 95 to 112% for DHA. These data are shown in Table 8.

3.9. Bench stability

Extracted validation samples at four QC levels were kept at room temperature for over 24 and 48 h and were reanalyzed and quantified against freshly made standard curves. The result show a considerable reduction in levels of ARTS and DHA when compared with samples analyzed at time zero. Data for the percentage reduction in levels of ARTS and DHA from baseline (0 h) are shown in Table 9.

3.10. Application to clinical sample analysis

The method was applied to a clinical study of ARTS in healthy volunteers. A oral dose of 2 mg/kg ARTS was administered to the volunteer. Thirteen samples were collected over a period of 12 h and analyzed using the proposed method. The sample run was analyzed in a batch of 31 samples which include 13 patient samples, 10 calibration levels and 8 QC's. QC's were run at the start and at the end of the run. Accuracy for QC's for ARTS and DHA were within a range of 90–102% and 105–110%, respectively. The plasma concentration versus time profile for a volunteer is shown in Fig. 5.

Table 8
Freeze–thaw precision and accuracy for ARTS and DHA in human plasma

	Theoretical concentration (ng/mL)			
	50	300	1300	2600
ARTS				
Overall mean (n = 5)	49.5	345	1421	2576
S.D.	1.4	31.7	187.7	46.95
CV (%)	2.8	9.2	13.2	1.8
DMT (%)	−1.0	16	9.3	−0.93
DHA				
Overall mean (n = 15)	51.5	284.8	1451	2597
S.D.	1.7	46.6	213.5	66.2
CV (%)	.3.3	15	14.6	2.5
DMT (%)	3.1	−5.1	11.6	−0.2

S.D., standard deviation; CV, coefficient of variation; DMT, deviation of mean value from nominal.

Table 9
Bench stability for ARTS and DHA in human plasma

	Theoretical concentration (ng/mL)			
	50 (%)	300 (%)	1300 (%)	2600 (%)
0 h				
ARTS (n = 5)	100	100	100	100
DHA (n = 5)	100	100	100	100
24 h				
ARTS (n = 5)	63.2	70.2	77.1	88.2
DHA (n = 5)	65.3	73.4	76.6	80.9
48 h				
ARTS (n = 5)	47.3	68.9	65.4	90.9
DHA (n = 5)	54.6	78.2	61.2	77.1

Values presented are percent deviation from baseline (time = 0 h); n, number of replicates.

4. Discussion

Several columns were investigated at the outset of this project to identify a suitable column and mobile phase to optimize the chromatography. The Synergi 4u Max-RP 80A was found to give sharp, symmetrical peaks and good sensitivity using a mobile phase consisting of acetic acid (0.1%): methanol: acetonitrile (38:46.5:15.5, v/v). Various other columns were also evaluated, but the Synergi Max-RP column gave the best separation and chromatography under the conditions tested.

Different mobile phase compositions were evaluated to increase the sensitivity, obtain better separation and sharper peaks. Formic acid and trifluoroacetic acid in the mobile phase were evaluated, but the separation and sensitivity was not as good as obtained with present acetic acid. To see whether pH has an effect on elution and sensitivity of the peaks, the pH of aqueous phase was varied using ammonium acetate, sodium acetate and ammonium formate. Poor separation between the parent and the metabolite was obtained in the pH range of 2.3–6.5.

Artemisinin was found to be suitable internal standard because its structure was similar to artesunate, and it was well recovered from plasma. Artemisinin had a retention time which lies between that of α-DHA and ARTS.

Analysis was tested with both positive and negative mode, but the sensitivity obtained with a positive mode was much higher then that of negative mode. Atmospheric pressure chemical ionization (APCI) source was used instead of electro spray ionization (ESI) because it gave a signal which was 10 times greater than that obtained from an ESI source.

The solid phase extraction method used was similar to that used by Batty and Davis [5]. However, we used Oasis HLB SPE Cartridges and they used bond-elute phenyl SPE cartridges for sample extraction. In our hands 40% ethyl acetate in butyl chloride solution was superior for elution rather than a 20% solution. An elution volume of 2 mL gave the highest recovery.

The mass detector response was linear over the range of 1–600 and 600–3000 ng/mL. Linear regression analysis performed both for ARTS and DHA at lower and higher concentration ranges yielded mean correlation coefficients ($n = 5$) of 0.996 and 0.994 at lower levels and 0.996 and 0.996 at higher levels for both ARTS and DHA, respectively. We believe this calibration curve range is appropriate for analyzing the body

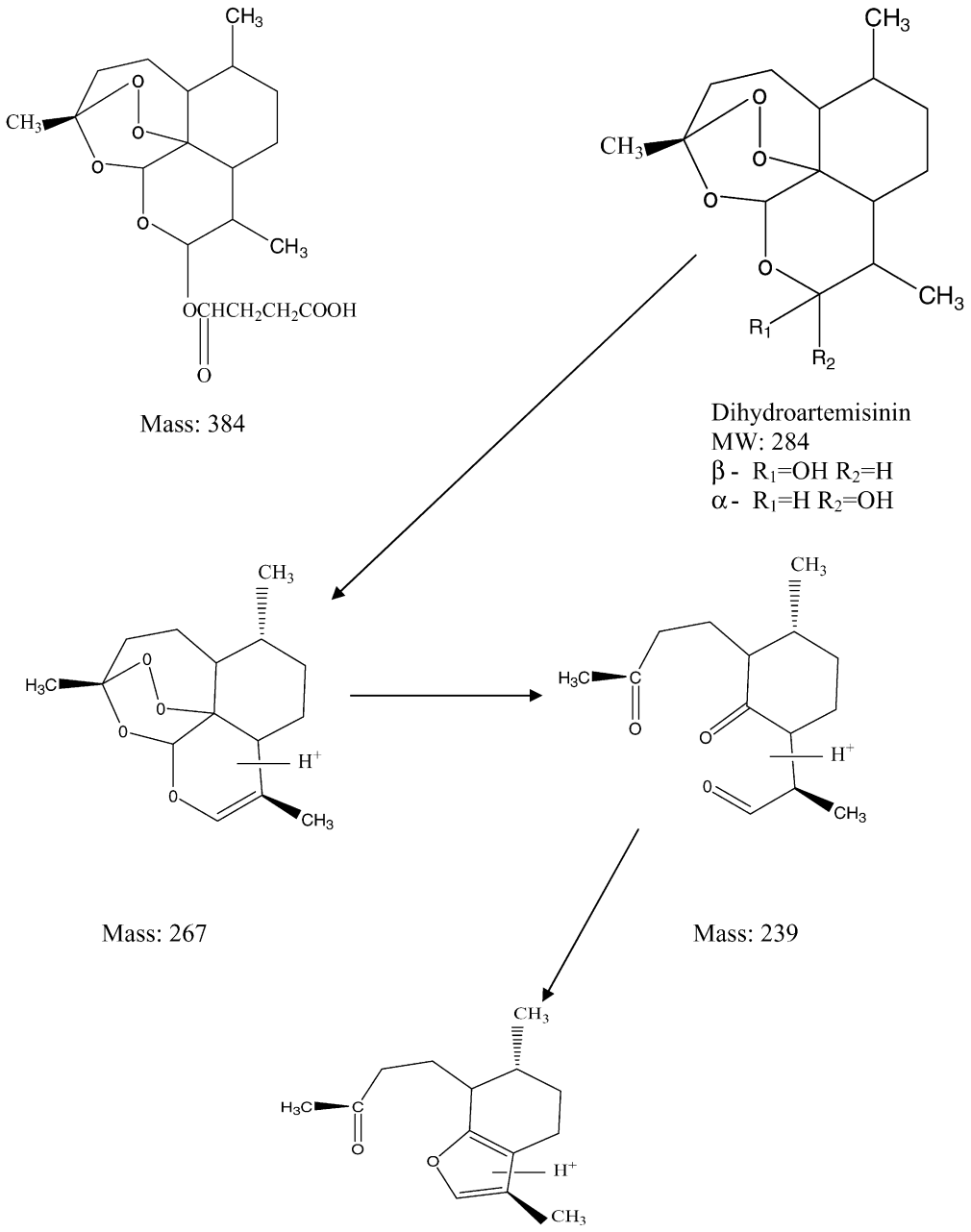

Fig. 4. Hypothetical fragmentation pattern for the formation of *m/z* 221 mass ion [10].

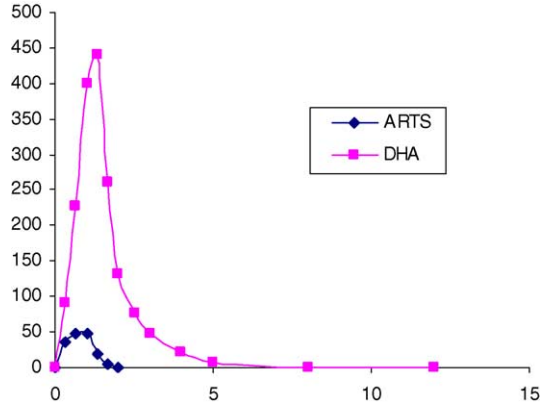

Fig. 5. Plasma concentration versus time profiles of ARTS and DHA after oral administration of 2 mg/kg ARTS.

fluids of subjects given anticipated ARTS doses. The quantification limit for artesunate was 1 ng/mL of plasma using an injection volume of 25 μL.

Analysis was done using a fragment ion of the parent compound instead of parent mass ion as sensitivity of the fragment ion was much greater than that of parent mass ion. A hypothetical fragmentation scheme to show the fragmentation pattern for the formation of *m*/*z* 221 fragment ion from the parent compound is shown in Fig. 4.

Validation of our method consisted of intra and inter-day reproducibility at four concentrations levels: 50, 300, 1300, 2600 ng/mL ($n = 5$). These concentrations were selected to cover entire range of the calibration curve. The intra-day and inter-day precision for plasma for all levels varied from 7% to 14% and 9% to 14% for ARTS and 11% to 14.9% and 11% to 15% for DHA, respectively. The variation was within the acceptable range required for validation of an assay [9].

A stability study was conducted to determine the storage temperature for plasma samples with minimum degradation. The study was conducted at three levels of concentration at an interval of 40 and 60 days. The analysis shows that the samples were stable up to 60 days. Therefore, all plasma samples were stored at −80 °C.

Freeze–thaw analysis results suggested that plasma samples could be thawed and refrozen without compromising the integrity of the sample. Bench stability analysis demonstrated that samples are not stable when kept on bench for 24 or 48 h.

5. Conclusion

We describe a sensitive and selective LC–MS assay procedure using solid phase extraction for the specific and quantitative analysis of ARTS and DHA in human plasma. The assay uses artemisinin as an internal standard, the extraction procedure is simple and relatively short allowing sufficient sample throughput to be applied to clinical pharmacokinetic studies of ARTS. The assay has been validated, and the results of validation show the method is reproducible and accurate. The analysis requires only 0.5 mL of plasma which is an advantage in pharmacokinetic studies. This LC–MS method has a detection limit of 1 ng/mL for the analytes of interest which is superior to other analytical methodologies.

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