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A SENSITIVE LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRIC ASSAY FOR THE DETERMINATION OF ARTEMETHER AND ITS ACTIVE METABOLITE DIHYDROARTEMISININ IN HUMAN PLASMA

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ABSTRACT

This paper describes a simple, rapid and sensitive liquid chromatography / tandem mass spectrometry (LC–MS/MS) assay method for the simultaneous quantification of artemether and its active metabolite, dihydroartemisinin in human plasma samples. Nevirapine was used as an internal standard. Analytes and the internal standard were extracted from 200 µL of human plasma *via* liquid-liquid extraction technique using *tert*-butyl methyl ether. The chromatographic separation was achieved on a C_{18} column by using a mixture of methanol and 5mM ammonium acetate buffer (90:10, v/v) as the mobile phase at a flow rate of 0.85 mL/min. The calibration curve obtained were linear ($r^2 \geq 0.99$) over the concentration range of 1.00-204.50 ng/mL for artemether and 0.51-161.52 ng/mL for dihydroartemisinin. Method validation was performed as per US FDA guidelines and the results met the acceptance criteria. A run time of 3.5 min for each sample made it possible to analyze more number of plasma samples per day. The proposed method can be applicable to clinical studies in humans.

Key words: Artemether and dihydroartemisinin; Liquid–liquid extraction (LLE); Liquid chromatography–tandem mass spectrometry (LC–MS/MS); Method validation.

INTRODUCTION

Malaria is one of the most popular infectious diseases in the world. Artemisinin is a natural anti-malarial derived from the Chinese medicinal plant *Artemisia annua*. The artemisinin derivative, artemether is a most effective anti-malarial drug available today for the treatment of multi-drug resistant strains of *falciparum malaria* [1-6]. Artemether give a rapid reduction in parasite biomass and consequently a rapid resolution of symptoms. The drug is highly effective against the blood schizonts of both malarial parasites P. falciparum and P. vivax [7]. It is available as mono-therapy but usually administred in combination with lumefantrine in clinical treatments of malaria.

As per the literature, numerous LC-MS methods have been reported for the determination of artemether and dihydroartemisinin in biological samples. The major disadvantages of these methods include, less sensitivity [8-15], requiring more sample volume [11, 13, 15], longer chromatographic run time [9, 11, 12, 14], complex and multi step extraction procedure [8, 10, 11] and narrow linearity range [12].

The method reported by Duthaler *et al.*, [9] and Hodel *et al.*, [12] employs protein precipitation (PP) method for the sample preparation. PP is most likely to cause ion suppression, since this method fails to sufficiently remove the endogenous compounds such as lipids, phospholipids, fatty acids, etc. Moreover the method proposed by Duthaler *et al.*, [9] in sheep plasma and

Magalhães *et al.*, [10] in rat plasma samples may not be applicable to pharmacokinetic/bioequivalence studies in humans. The another method proposed by Souppart *et al.*, [13] and Peys *et al.*, [15] described a single–quadrupole mass spectrometry (LC–MS) with selected–ion monitoring (SRM) mode to detect the precursor ion. But in the present method, a triple–quadrupole mass spectrometry (LC–MS/MS) with MRM mode was used to detect both the precursor ion and product ion. It shows that the proposed method is highly specific.

To overcome above drawbacks, in the present work we have developed and validated a high–throughput LC–MS/MS method for the quantitation of artemether and its active metabolite dihydroartemisinin in 200 µL of human plasma with an LLOQ of 1.00 ng/mL and 0.51 ng/mL, respectively. We have also employed simple liquid–liquid extraction (LLE) technique for sample preparation to get cleaner extracts for the analysis and used commercially available nevirapine as an internal standard (IS).

EXPERIMENTAL

Standards and chemicals: The reference samples of artemether (≥98% pure), dihydroartemisinin (≥97% pure) and nevirapine (≥98% pure) were obtained from Sigma Aldrich (Bangalore, India). Their structures are shown in Fig. 1. HPLC grade methanol and *tert*-butyl methyl ether (TBME) were purchased from J.T. Baker (Phillipsburg, USA). Analytical grade ammonium acetate was purchased from Merck Ltd (Mumbai, India). GR grade ammonia solution was also purchased form Merck Ltd (Mumbai, India). Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). The control human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS/MS instrument and conditions: An HPLC system (Shimadzu, Kyoto, Japan) consisting of an ACE C_{18} column (100 mm × 4.6 mm, 5 µm), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) was used for the study. An aliquot of 15 µL of the processed samples were injected into the column, which was kept at 40 ºC. An isocratic mobile phase consisting of a mixture of methanol and 5mM ammonium acetate (90:10, v/v) was used to separate the analytes from the endogenous components and delivered at a flow rate of 0.85 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode for the analytes and the IS using an MDS Sciex API–4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 450 ºC. The ion spray voltage was set at 5500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 42, 40, 25, and 5 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 27, 10, 10, 20 V for artemether and 32, 10, 10, 20 V for dihydroartemisinin and 50, 10, 10, 25 V for the IS. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 316.2 precursor ion to the *m/z* 267.2 for artemether, *m/z* 302.3 precursor ion to the *m/z* 267.1 for dihydroartemisinin and *m/z* 267.1 precursor ion to the *m/z* 226.1 product ion for the IS. Quadrupoles Q1 and Q3 were set at unit resolution. The analysis data obtained were processed by Analyst Software™ (version 1.4.2).

Preparation of plasma standards and quality controls: Standard stock solutions of artemether, dihydroartemisinin and the IS (1 mg/mL) were prepared in methanol. Working solutions for calibration curve standards and quality control samples were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (50 ng/mL) was prepared by diluting its stock solution with diluent. All the stock solutions were found to be stable for 20 days at 2–8 °C (data not shown).

Calibration samples were prepared by spiking 950 µL of control human plasma with the appropriate working standard solution of the each analyte (25 µL of artemether and 25 µL of dihydroartemisinin) as a bulk, giving the final concentrations of 1.00, 2.01, 5.02, 20.09, 40.17, 80.35, 119.92, 160.54 and 204.50 ng/mL for artemether and 0.51, 1.01, 6.06, 12.11, 24.23, 48.46, 96.91, 129.21 and 161.52 ng/mL for dihydroartemisinin.

The CC samples were analyzed along with the quality control (QC) samples for each batch of plasma samples. The QC samples were prepared at five different concentration levels of 1.01 (lower limit of quantitation, LLOQ), 3.01 (low quality control, LQC), 30.11 (middle quality control, MQC–1), 100.35 (MQC–2) and 171.25 (high quality control, HQC) ng/mL for artemether and 0.51 (LLOQ), 1.55 (LQC), 20.87 (MQC–1), 83.50 (MQC–2) and 141.52 (HQC) ng/mL for dihydroartemisinin in blank plasma. All the prepared plasma samples were stored at –70 ± 10 °C.

Sample processing protocol: All frozen calibration curve standards and quality control samples were thawed and allowed to equilibrate at room temperature prior to analysis. The samples were vortexed to mix for 10 s prior to spiking. A 200 µL aliquot of human plasma sample was mixed with 50 µL of the internal standard working solution (50 ng/mL of nevirapine). To this, 50 µL of 0.1% ammonia solution was added. After vortexing for 15 s, a 4 mL of *tert*–butyl methyl ether was added using Dispensette Organic (Brand GmbH, Wertheim, Germany). The sample was shaken for 10 min using a reciprocating shaker (Scigenics Biotech, Chennai, India) and then centrifuged for 5 min at 4000 rpm on Megafuse 3SR (Heraeus, Germany). The clear organic layer (3 mL) was transferred to a 5 mL glass test tube and evaporated at 40°C under a gentle stream of nitrogen. The dried extract was reconstituted with 200 µL of the mobile phase and a 15 µL aliquot of it was injected into the LC–MS/MS system.

Bioanalytical method validation: The validation of the above method was carried out as per US FDA guidelines (US DHHS, *et al*., 2001) [16]. The parameters determined were selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability. Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources including one lipemic and hemolyzed plasma. Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations. Matrix effect was checked with six different lots of K_2 EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC samples in total). For checking the linearity standard calibration curves containing at least 9 points (non–zero standards) were plotted. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. To determine intra–day accuracy and precision, a calibration curve and six replicates of LLOQ QC, LQC, MQC–1, MQC–2, and HQC were analysed on the same day. Inter–day accuracy and precision were assessed by analyzing three batches of samples on two consecutive days. Recovery of analytes and the IS were determined by comparing the peak area of extracted analyte standard with the peak area of non–extracted standard. Recoveries of artemether and dihydroartemisinin were determined at a concentration of 3.01, 1.55 (LQC), 100.35, 83.50 (MQC–2) and 171.25, 141.52 (HQC) ng/mL, respectively, whereas for the IS was determined at concentration of 50 ng/mL. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates each at a concentration of about 1.6 times of the uppermost calibration curve standard were diluted two– and four–fold with blank plasma. The diluted samples were processed and analyzed.

Stability tests were conducted to evaluate the analytes stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8 °C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (12 h), processed samples stability (autosampler stability for 43 h, wet extract stability for 38 h and reinjection stability for 25 h), freeze–thaw stability (four cycles), long–term stability (45 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (≤15% RSD).

RESULTS AND DISCUSSION

Mass spectrometry: During method development, mass parameters were tuned in positive and negative ionization modes using electrospray ionization source for the analytes and the IS at a concentration of 100 ng/mL tuning solution. However, the response observed was much higher in positive ionization mode for the analytes and the IS compared to the negative mode due to their basic nature. Moreover, use of 5mM ammonium acetate in the mobile phase further enhanced the detection of analytes and the IS with low background noise, resulting in higher sensitivity. Protonated form of analyte and IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from *m/z* 316.2 to 267.2 for artemether, from *m/z* 302.3 to 267.1 for dihydroartemisinin and from *m/z* 267.1 to 226.1 for the IS. The dwell time for each transition was 200 ms. As earlier publications have discussed the details of fragmentation patterns of artemether, dihydroartemisinin and the IS [11, 14, 17], we are not presenting the data pertaining to this. The inherent selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. The LC–MRM technique was chosen for the assay development due its inherent selectivity and sensitivity [18].

Method development: The objective of the present work was to develop and validate a LC–MS/MS method for the simultaneous determination of artemether and active metabolite dihydroartemisinin in human plasma samples with high sensitivity to monitor their concentrations for pharmacokinetic/bioequivalence studies. For a sensitive and selective analytical method requires the judious selection of chromatographic conditions such as analytical column, mobile phase composition and type of organic solvent. These parameters should be carefully monitored to produce the required resolution from endogenous components which in turn affect sensitivity and reproducibility of the analytical method by ion suppression. Once above conditions are set then flow rate, column temperature and buffer type and concentration can be manipulated for finest response.

Chromatographic separation was attempted using organic solvents such as acetonitrile and methanol in different volume ratios with buffers like ammonium acetate, ammonium formate (5–15 mM) as well as acid additives like acetic acid and formic acid (0.1–0.5%) in varying strength of each component on different columns like C_8 and C_{18} of different makes (Ace, Hypersil BDS, Zorbax SB C_{18}, Inertsil ODS, Grace C_{18}, Kromasil C_{18}, etc). It was observed that methanol and 5mM ammonium acetate buffer (90:10, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. ACE C_{18} column (100 mm × 4.6 mm, 5 µm) gave good peak shape and response even at lowest concentration level of the analytes. In addition, the flow rate was monitored from 0.3 to 1.0 mL/min and finally set at 0.85 mL/min. The retention time of artemether, dihydroartemisinin and the IS were low enough (2.5, 1.6 and 1.4 min) allowing a chromatographic run time of 3.5 min.

At the initial stage of the work, both the extraction methodologies solid phase extraction (SPE) and protein precipitation (PP) were tested using Oasis HLB and Orpheus C18 cartridges for SPE and different organic solvents like ethanol, methanol and acetonitrile under acidic conditions for PP. However, the response was inconsistent for both the analytes especially at the LLOQ level due to ion suppression with PP. The promising results were obtained with Oasis HLB with cartridges at all QC levels for the analytes. But as the purpose was to develop a simple, rapid and sensitive method with minimum or no matrix effect, LLE was tested. LLE is helpful in producing a clean sample and avoiding the introduction of non–volatile materials onto the column and MS system and also minimizing the experimental cost. Clean samples are essential for minimizing ion suppression and matrix effect on LC–MS/ MS. hence, LLE was carried out using solvents like diethyl ether, *tert*–butyl methyl ether, ethyl acetate, hexane, and dichloromethane alone and in combination under acidic or basic conditions. Among different solvents checked for their suitability, consistent and quantitative recovery was obtained with *tert*–butyl methyl ether at all QC levels for the analytes. Addition of ammonia solution to the plasma samples as an extraction additive helped in achieving the higher and reproducible recoveries.

An ideal internal standard should mimic the analyte in as many ways as possible. It should have a similar structure, same physicochemical properties or can be a labeled compound. Isotope–labeled drugs or deuterated compounds are preferable for LC-MS/MS analysis. But these compounds are expensive and/or not available to serve as IS. Hence, the different compounds were checked for their suitability to serve as internal standard and finally nevirapine was found to be best for the present purpose.

Selectivity and chromatography: The degree of interference by endogenous plasma constituents with the analytes and the IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Figs. 2 and 3, no significant direct interference in the blank plasma traces was observed from the endogenous substances in drug–free plasma at the retention time of the analytes and the IS.

Sensitivity: The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 11.27% and 95.58% and 5.74% and 83.83% for artemether and dihydroartemisinin, respectively.

Matrix effect: No significant matrix effect was observed in all the six batches of human plasma for the analytes at low and high quality control concentrations. The precision and accuracy for artemether at LQC concentration were found to be 2.49% and 102.80%, and at HQC level they were 1.77% and 95.81%, respectively. Similarly, the precision and accuracy for dihydroartemisinin at LQC concentration were found to be 0.69% and 93.36%, and at HQC level they were 0.59% and 89.34%, respectively.

Linearity: The nine–point calibration curve was found to be linear over the concentration range of 1.00-204.50 ng/mL for artemether and 0.51-161.52

for dihydroartemisinin. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥ 0.99 for both the analytes.

Precision and accuracy: The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Table 1. The intra–day and inter day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle and high quality control levels, whereas within 20% at LLOQ QCs level. The intra–day and inter–day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle and high quality control levels, whereas within 100± 20% at LLOQ QCs level. The results revealed good precision and accuracy.

Extraction efficiency: Simple liquid–liquid extraction with TBME proved to be robust and provided the cleanest samples. The recovery of the analytes and the IS were good and reproducible. The mean overall recovery (with the precision range) of artemether and dihydroartemisinin were 50.19 ± 1.14% (0.18–4.16%) and 45.60 ± 1.07% (1.84–4.29%), respectively. The recovery of the IS was 71.56% with a precision range of 1.78–3.41%.

Dilution integrity: The upper concentration limits can be extended to 329.20 ng/mL for artemether and 260.01 ng/mL for dihydroartemisinin by 2–fold and 4–fold dilutions with screened human blank plasma. The mean back calculated concentrations for 2–fold and 4–fold dilution samples were within 85–115% of their nominal value. The coefficients of variation (%CV) for 2–fold and 4–fold dilution samples were less than 7%.

Stability studies: In the different stability experiments carried out viz. bench top stability (12 h), autosampler stability (43 h), repeated freeze–thaw cycles (4 cycles), reinjection stability (25 h), wet extract stability (38 h at 2–8 °C) and long term stability at –70 °C for 45 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2). Thus, the results were found to be within the acceptable limits during the entire validation.

CONCLUSIONS

The LC–MS/MS assay presented in this paper is simple, rapid, specific and sensitive for quantification of artemether and its active metabolite dihydroartemisinin in human plasma and is fully validated according to commonly acceptable FDA guidelines. The simple liquid-liquid extraction method with TBME gave consistent and reproducible recoveries for the analytes from plasma samples. The method provided good linearity. A sample turnover rate of less than 3.5 min makes it an attractive procedure in high–throughput bioanalysis of artemether and its active metabolite dihydroartemisinin. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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COMPETING INTERESTS

The authors declare no conflict of interest.

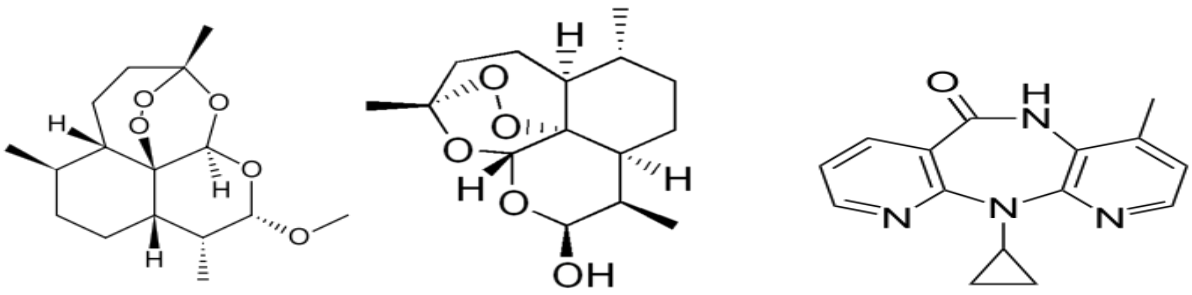
Table 1. Precision and accuracy data for artemether and dihydroartemisinin in human plasma samples.

Analyte	Concentration added (ng/mL)	Intra-day precision and accuracy (*n*=12; 6 from each batch)			Inter-day precision and accuracy (*n*=18; 6 from each batch)		
		Concentration found (mean ± S.D; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D; ng/mL)	Precision (%)	Accuracy (%)
Artemether	1.01	1.01 ± 0.07	6.97	99.93	0.99 ± 0.06	6.10	98.36
	3.01	3.11 ± 0.11	3.42	103.15	3.04 ± 0.11	3.56	100.98
	30.11	32.72 ± 1.18	3.60	108.69	31.65 ± 1.54	4.87	105.13
	100.35	101.04 ± 3.22	3.18	100.69	99.15 ± 2.91	2.94	98.80
	171.25	167.52 ± 6.20	6.20	97.82	167.01 ± 5.74	3.44	97.52
Dihydroartemisinin	0.51	0.45 ± 0.03	6.17	88.00	0.45 ± 0.03	6.73	88.31
	1.55	1.43 ± 0.06	4.39	92.79	1.42 ± 0.06	4.01	91.72
	20.87	20.84 ± 0.44	2.12	99.84	20.82 ± 0.39	1.86	99.73
	83.50	81.82 ± 1.19	1.46	98.11	81.87 ± 1.40	1.71	98.05
	141.52	141.41 ± 1.14	0.81	99.92	141.22 ± 1.11	0.79	99.79

Table 2. Stability data for artemether and dihydroartemisinin in human plasma samples (*n*=6).

Analytes	Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Artemether	Process[a]	3.01	3.02 ± 0.05	100.46	1.62
		171.25	161.74 ± 2.10	94.45	1.30
	Process[b]	3.01	3.00 ± 0.06	99.66	2.16
		171.25	160.93 ± 2.46	93.97	1.53
	Bench top[c]	3.01	3.11 ± 0.06	103.38	1.95
		171.25	160.75 ± 1.62	93.87	1.01
	FT[d]	3.01	3.04 ± 0.04	100.80	1.27
		171.25	161.83 ± 1.47	94.50	0.91
	Reinjection[e]	3.01	3.13 ± 0.08	103.82	2.58
		171.25	170.80 ± 3.80	103.23	2.22
	Long-term[f]	3.01	2.96 ± 0.12	98.17	4.00
		171.25	169.84 ± 3.37	99.17	1.99
Dihydroartemisinin	Process[a]	1.55	1.41 ± 0.03	91.73	1.84
		141.52	124.67 ± 0.99	88.68	0.79
	Process[b]	1.55	1.41 ± 0.02	92.14	1.72
		141.52	124.24 ± 1.65	88.38	1.33
	Bench top[c]	1.55	1.42 ± 0.02	92.48	1.22
		141.52	124.60 ± 1.46	88.63	1.17
	FT[d]	1.55	1.44 ± 0.02	93.80	1.61
		141.52	125.43 ± 0.71	89.23	0.57
	Reinjection[e]	1.55	1.35 ± 0.04	87.51	3.20
		141.52	134.20 ± 1.47	94.83	1.09
	Long-term[f]	1.55	1.46 ± 0.12	94.53	8.49
		141.52	143.83 ± 2.13	101.63	1.48

[a] after 43 h in autosampler at 10°C; [b] after 38 h in refrigerator at 2-8°C; [c] after 12 h at room temperature; [d] after four freeze and thaw cycles; [e] after 25 h of Reinjection; [f] at -70°C for 45 days



Artemether Dihydroartemisinin Nevirapine (IS)

Figure 1. Chemical structures of artemether, dihydroartemisinin and neverapine (IS).

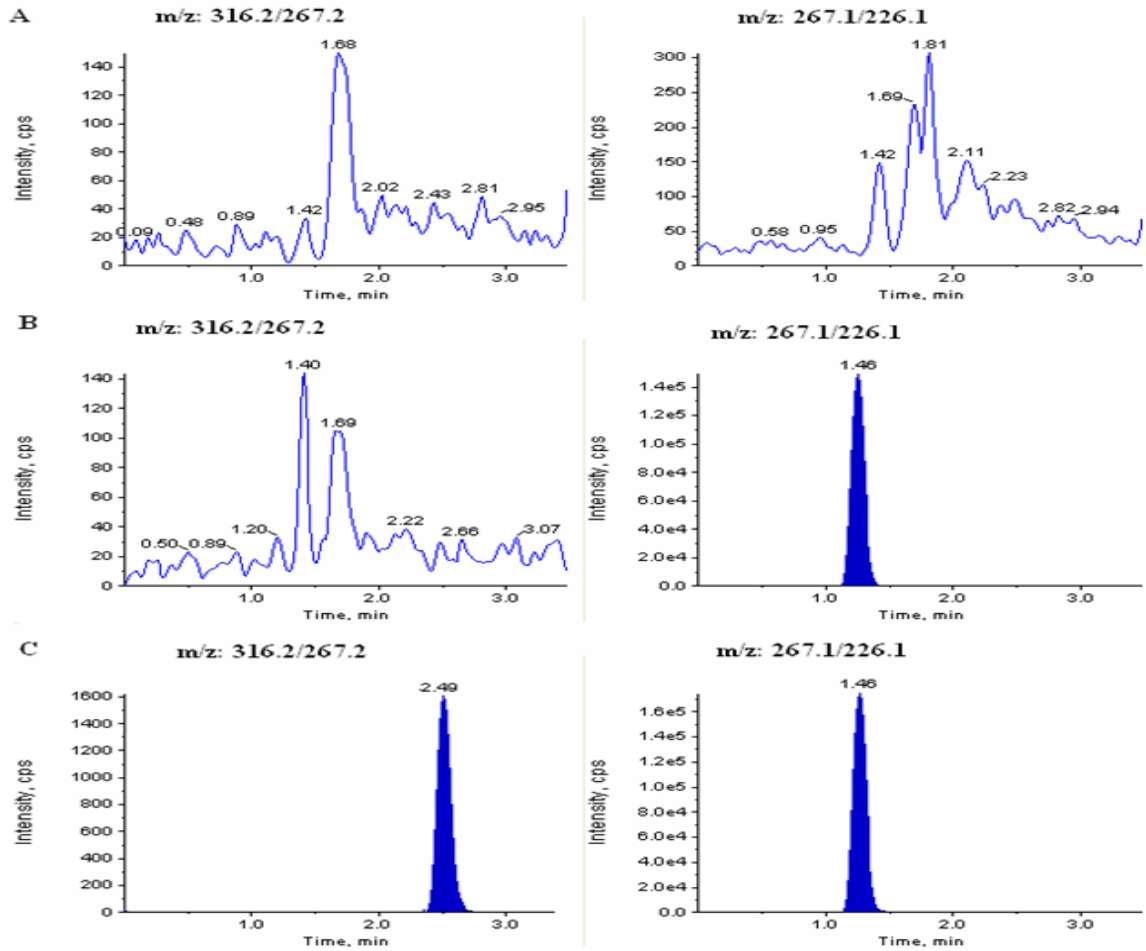


Figure 2. Typical MRM chromatograms of artemether (left panel) and IS (right panel) in human blank plasma (A), human plasma spiked with IS (B), and a LLOQ sample along with IS (C).

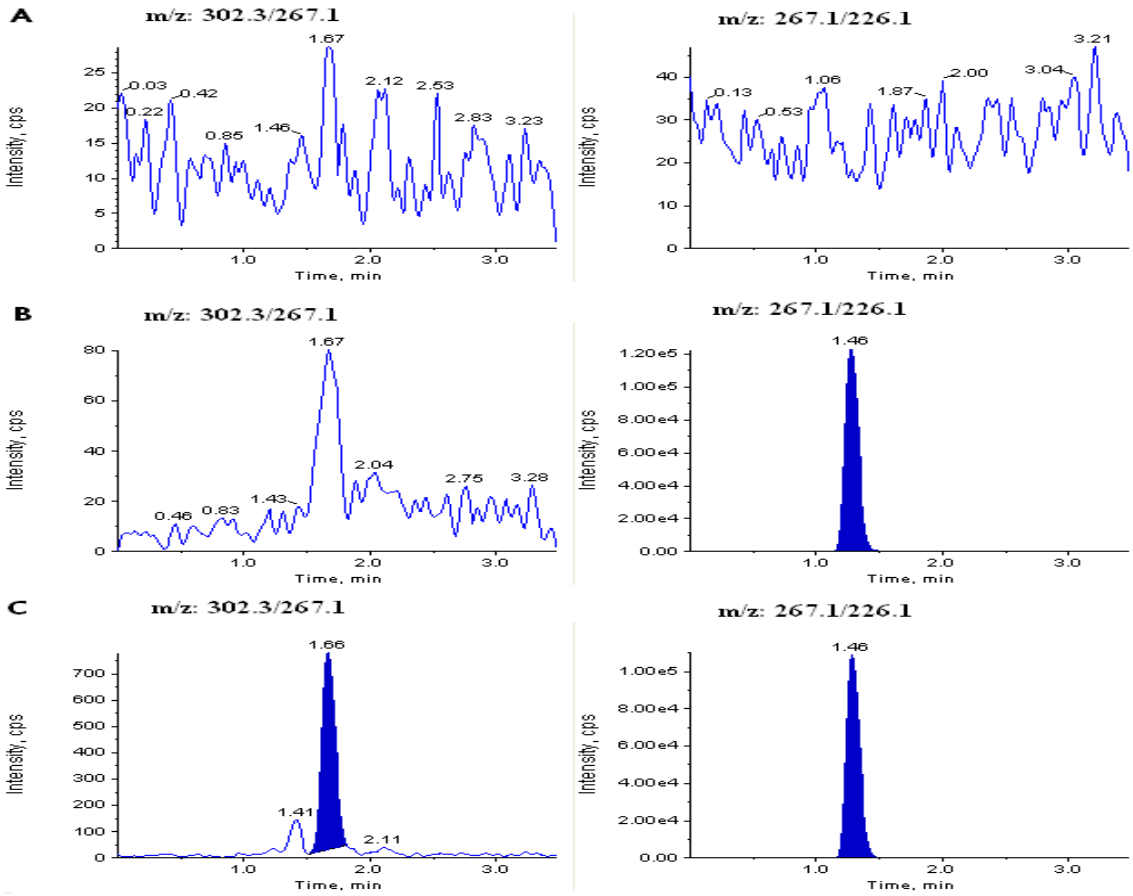


Figure 3. Typical MRM chromatograms of dihydroartemisinin (left panel) and IS (right panel) in human blank plasma (A), human plasma spiked with IS (B), and a LLOQ sample along with IS (C).

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