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A SENSITIVE AND ACCURATE METHOD FOR SIMULTANEOUS DETERMINATION OF ARTESUNATE AND ITS METABOLITE DIHYDRO- ARTEMISININ IN HUMAN PLASMA BY USING LC-MS/MS METHOD

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ABSTRACT
A sensitive, accurate, precise and rugged method was developed and validated by using LC-MS/MS for Artesunate (AS) and its metabolite dihydroartemisinin (DHA) with internal standards. The extraction procedure carried out by liquid-liquid extraction using t-BME solvent and separated on a Thermo, Hypersil BDS, C_{18} (100 mm × 4.6 mm, 5 µm) column. The mobile phase was composed of 2 mM Ammoniumformate (pH 2.5): Acetonitrile in the ratio of 15:85 as a mobile phase at a flow rate of 0.5 mL/min in isocratic with 2.5 mins of run time. Artesunate (AS) and metabolite dihydroartemisinin (DHA) were ionized in positive polarity and detected in the selective reaction monitoring mode (SRM) using m/z 402.124→163.006, m/z 302.244→163.186, and internal standards Artesunate D_3 and dihydroartemisinin D_4 (AS-D_3), (DHA-D_4) m/z 406.172→163.078 and m/z 305.189→166.159 respectively. The linearity range used for quantification is 5.000-500.000 ng/mL for Artesunate (AS) and 10.000-1000.000 ng/mL for dihydroartemisinin (DHA) respectively.

KEYWORDS: Artesunate, Dihydroartemisinin, Human plasma, Prodrug.

1. INTRODUCTION

WHO was recommended for Artesunate (AS) and Dihydroartemisinin (DHA)[1] were used as anti malarial drugs. In worldwide more than 1.5 million deaths were noticed due to malaria (Plasmodium falciparum). In recent years, there is an increase in the use of the drugs, because of their efficacy against multidrug-resistant falciparum malaria and their potential to delay anti malarial drug resistance when used in combinations with other mainly long acting antimalarial drugs like sulfamethoxypyrazine/pyrimethamine or amodiaquine. The Artesunate and its derivatives are found to be effective against malaria. Artesunate is a hemi-succinate ester and Dihydroartemisinin is the active metabolite of all artemisinin compounds (artemisinin, artesunate, artemether, etc.) fine, white crystalline powder and readily soluble in water.

Artesunate and Dihydroartemisininare also available as a drug in itself. It is a semi-synthetic derivative of artemisinin and is widely used as an intermediate in the preparation of other artemisinin-derived antimalarial drugs and their chemical structures are shown below Fig: 1 [a,b].

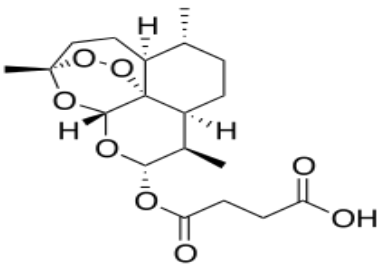
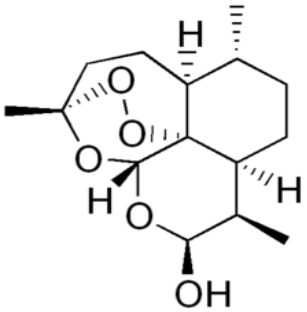


Fig-1: (A) Structure of Artesunate.



(B) Structure of Dihydroartemisinin.

Artesunate[2] is the first line treatment for children or adults with severe malaria. Mechanism of action of Artesunate is a prodrug that is rapidly converted to its active form dihydroartemisinin (DHA). This process involves hydrolysis of the 4-carbon ester group via plasma esterase enzyme. It is hypothesized that the cleavage of endoperoxide bridge in the pharmacophore of DHA generates reactive oxygen species (ROS), which increases oxidative stress and causes malarial protein damage via alkylation. In addition, Artesunate potently inhibits the essential Plasmodium falciparum exported protein 1 (EXP1), a membrane glutathione S-transferase. As a result, the amount of glutathione in the parasite is reduced.

In infected individuals, the elimination half-life of artesunate is about 0.22 hours. Its active metabolite, DHA, has a slightly longer half-life of 0.34 hours. Overall, the average half-life ranges from 0.5 to 1.5 hours. Because of its short half-life, its use in malaria prevention is limited.[3]

In 2016, artemisinin has been shown to bind to a large number of targets, suggesting that it acts in a promiscuous manner. There is evidence suggesting DHA inhibition of calcium-dependent ATPase on endoplasmic membrane, which disrupts protein folding of parasites.

Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) technique is generally accepted by for quantifying the small drugs, metabolites in human plasma and genotoxic impurities in active pharmaceuticals (APIs), since this technique is highly selective and sensitive.

Literature[4,8] review stated that only few LC-MS/MS studies has been reported for the quantification of Artesunate and dihydroartemisinin in human plasma with liquid-liquid extraction, protein precipitation and solid phase extraction method, whereas no analytical method has been reported for simultaneous determination of Artesunate and dihydroartemisinin by using tertiary-Butyl methyl ether as extraction solvent (t-BME). Therefore, now we developed a sensitive LC-MS/MS and accurate method for simultaneous determination of Artesunate and its metabolite dihydroartemisinin in human plasma by using a simple and economical liquid-liquid extraction method with tertiary-Butyl methyl ether (t-BME).

During method development and validation results are consistent, accurate, and precise and there are no major failures. Finally, the simultaneous estimation of AS, DHA method development involves simple LLE technique with electrospray ionization, selected reaction monitoring(SRM), higher sensitivity (5 ng/mL, 10 ng/mL for AS and DHA respectively), higher recovery, least matrix effect, the most stable, rugged, less run time and robust compared to the already available methods.[4,8]

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

The working standards Artesunate (AS), Dihydroartemisinin (DHA) and isotope labeled internal standards Artesunate D3 (ASD_3) and Dihydroartemisinin ($DHAD_4$) are free gift samples were obtained from Clear synth labs. HPLC grade solvents like Methanol, Acetonitrile from Fisher Scientific, Ammonium formate from Fisher Scientific, Formicacid from MERCK and HPLC grade water from RANKEM. Plasma was procured from Sri Lakshmi blood bank. (Hyderabad).

2.2 Solution preparation

Methanol: Water (50:50 v/v), Acetonitrile: Water (50:50 v/v), 2 mM Ammonium formate (pH 2.5):Acetonitrile (15:85 v/v) as a diluent, rinsing solution, mobile phase or reconstitution solution respectively.

2.3 Preparation of Calibration Curve Standards and Quality Control Samples

Stock solutions of AS, DHA and ASD_3 and $DHAD_4$ were weighed equivalent to 1 mg/mL and dissolved in methanol. Calibration curve standard and quality control samples were prepared in diluent with a planned linearity range of 5.000-500.000 ng/mL for AS, 10.000-1000.000 ng/mL for DHA. The spiking stock solutions were prepared by using freshly weighed and prepared working stock. The obtained nominal concentrations were applied over all the quantification experiments. Nominal concentrations after spiking into plasma for calibration standards and quality control samples were CC-AS-5.005, 10.012, 20.125, 50.007, 100.057, 200.092, 300.125, 400.034, 500.026 ng/mL, QC-AS-37501.95, 17501.91, 12.513, 5.007 ng/mL and CC-DHA-10.026, 20.063, 40.104, 100.042, 200.081, 400.126, 600.053, 800.114, 1000.034 ng/mL, QC-DHA-75002.550, 35002.160, 25.065, 10.028 ng/mL. Internal standard working solution is prepared by using diluent to obtain the concentration of 50 ng/mL and 100 ng/mL for ASD_3 and $DHAD_4$ respectively.

2.4 Sample preparation

Extraction of AS and DHA along with its internal standards achieved through liquid-liquid extraction technique. The extraction procedure involved in various steps which start with, aliquoting 200 µL of spiked plasma into processing tubes, add 25 µL of cocktail internal standard solution (200 ng/mL concentrations of ASD_3 and $DHAD_4$) were added and mixed well. 4.00 mL of extraction solvent t-BME (tertiary-Butyl methyl ether) was added and vortex it for 10 min at 2500 rpm. The samples were centrifuged for 10 mins at 10°C, 5000 rpm. After completing the centrifugation process, the supernatant solutions from each tube were collected into prelabeled tubes to evaporate under nitrogen stream at 40°C. The evaporated samples were reconstituted with

500 µL of reconstitution solution and mixed well before loading the reconstituted sample into injector vials.

2.5 Instrumentation and conditions

LC system was an Agilent 1200 series with binary pumping system, autosampler and column oven compartments were equipped with temperature controllers. Thermo, Hypersil BDS, C_{18} (100 mm × 4.6 mm, 5 µm) column was used for chromatographic separation in liquid chromatographic system with 2.5 mins of runtime. The tandem mass spectrometry system Thermo Quantum, Ultra and software version 2.6.4 were used for detection by using HESI ion source housing in positive polarity with selective reaction monitoring mode (SRM). The transitions used for analytical method were AS m/z 402.124 → 163.006, ASD_3 m/z 406.172→163.078 and DHA m/z 302.244→163.186 $DHAD_4$ m/z 305.189→166.159. Ion source based parameters, Spray voltage-5000 v, Vaporizer temperature-400°C, Sheath gas pressure-40 Atmos, Auxiliary gas-25 Atmos, Capillary temperature-250, Polarity-positive, Data type- centroid. Compound dependent parameters Tube lens offset-60 (AS, ASD_3), 55 (DHA, $DHAD_4$), Skimmer offset- 10 (AS, ASD_3) and (DHA, $DHAD_4$), Collision energy (CE)-25 (AS, ASD_3) and (DHA, $DHAD_4$).

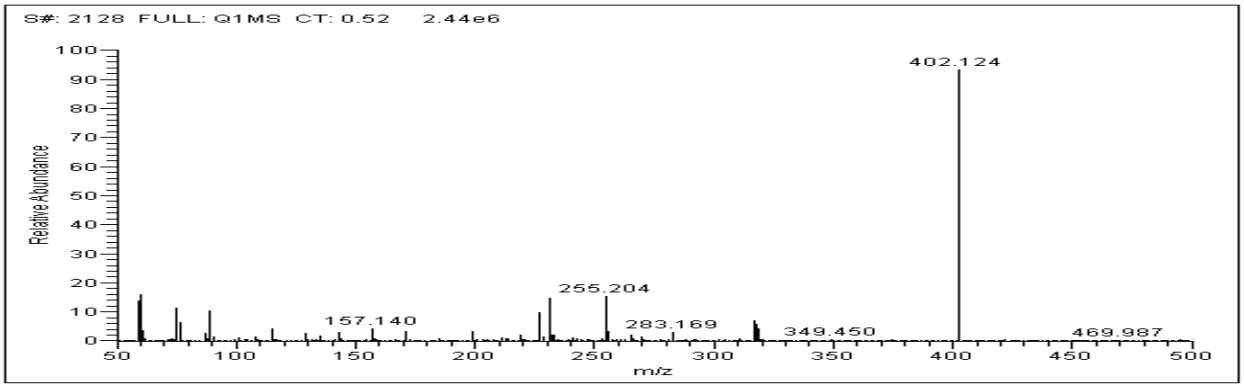


Fig-2: ESI-MS/MS Q1 spectra for Artesunate (AS).

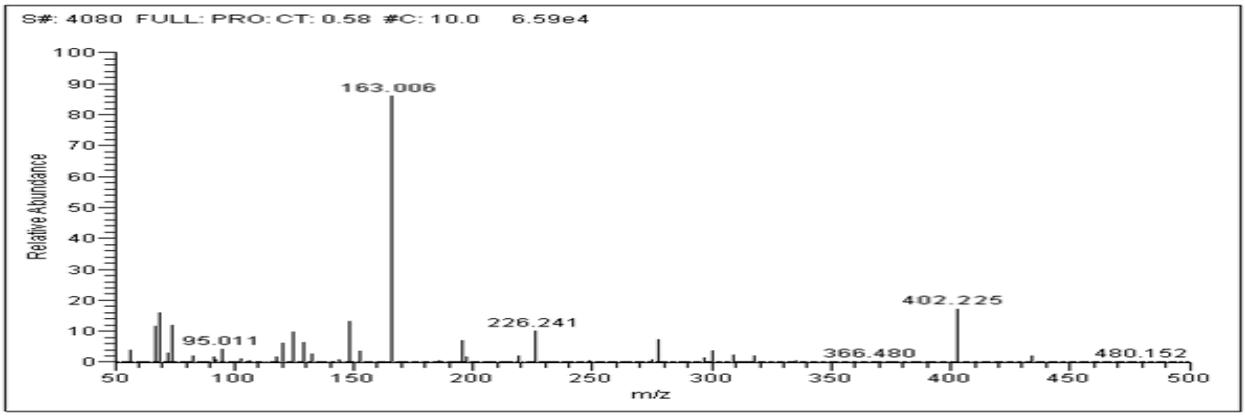


Fig-3: ESI-MS/MS Q3 spectra for Artesunate (AS).

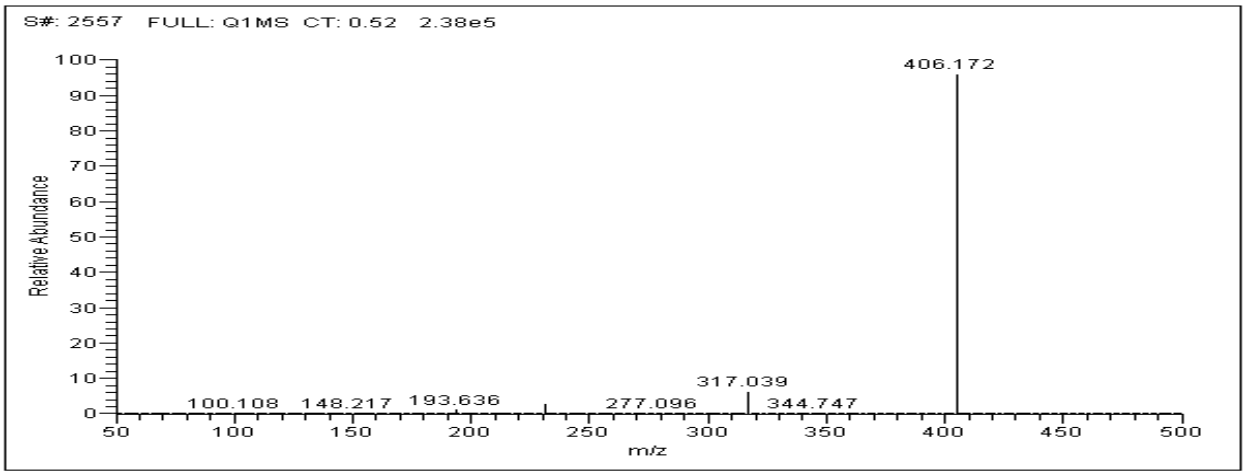


Fig-4: ESI-MS/MS Q1 spectra for Artesunate D_3 (ASD_3).

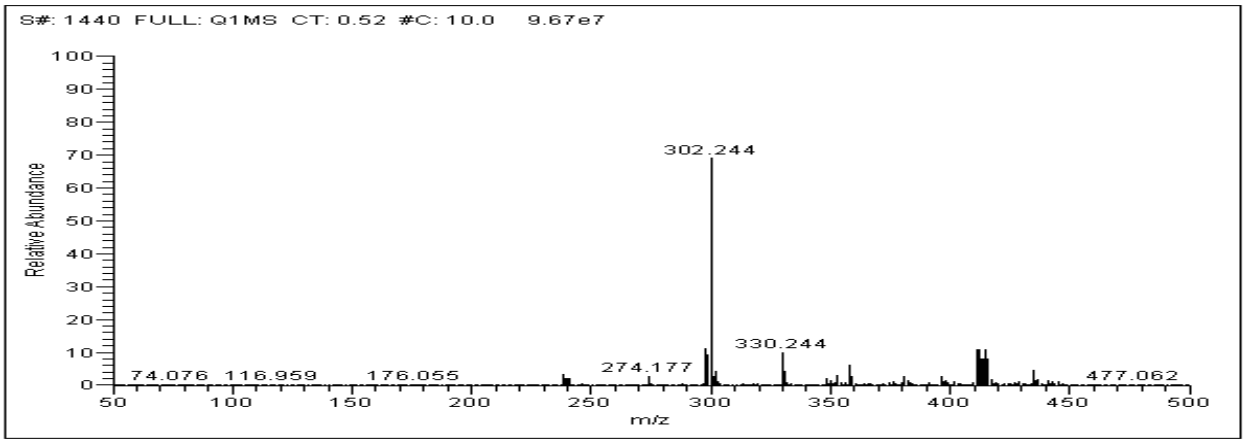


Fig-5: ESI-MS/MS Q1spectra for Dihydroartemisinin (DHA).

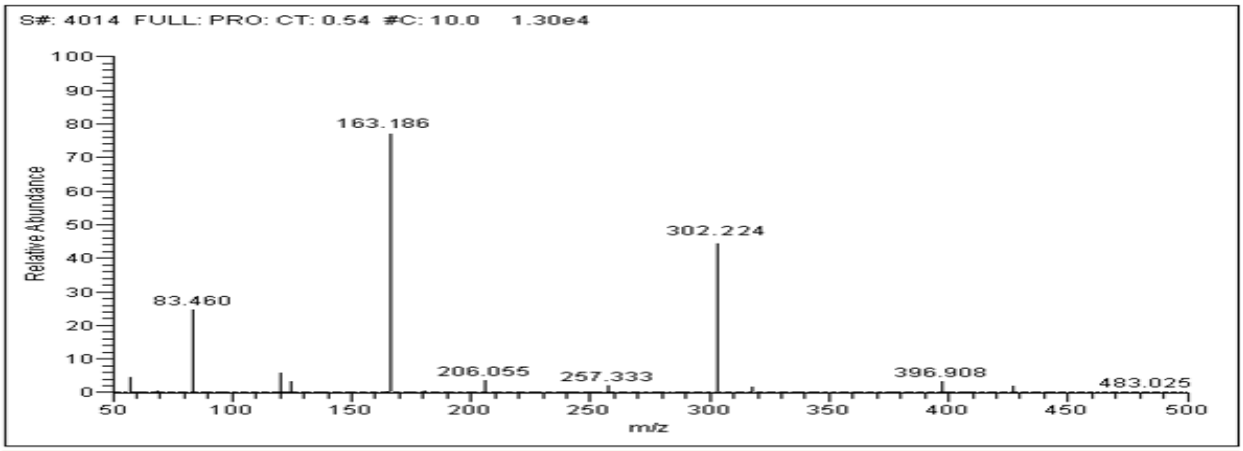


Fig-6: ESI-MS/MS Q3spectra for Dihydroartemisinin (DHA).

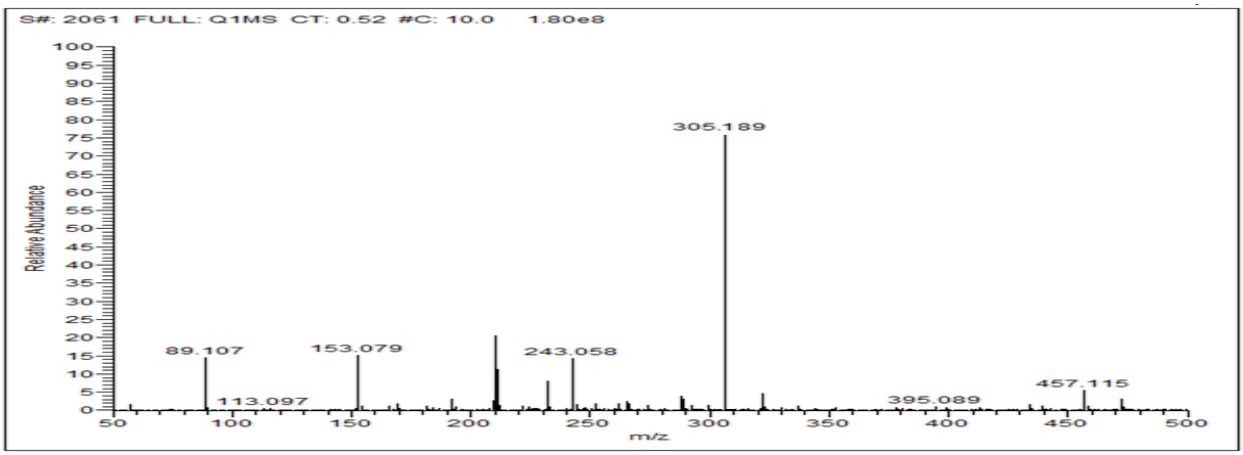


Fig-7: ESI-MS/MS Q1 spectra for Dihydroartemisinin D_4 ($DHAD_4$).

3. RESULTS AND DISCUSSION

3.1 Method development

3.1.1 Tuning and optimization

The working stocks were weighed equivalent to 1 mg/mL and dissolved in methanol. After getting the clear solution, concentrations of 50 ng/mL solutions were prepared for all the weighed stocks which include analyte, metabolite and internal standards. During tuning, the compound to the mass spectrometer one or two drops of diluted ammonia solution was added to the above prepared 50 ng/mL solution to get intensified spectra. During tuning it was observed that, Q1 (parent ion)/ protonated ion for all the compounds used for tuning resulted with cation NH_3^+ $(M+NH_3)^+$. This ammoniated cation adduct identified during the tuning in all the compounds was used for entire method development and method validation.

3.1.2 Extraction method

The present method development and validation was carried out in liquid-liquid extraction (LLE) method. The LLE method was based on distribution of solutes

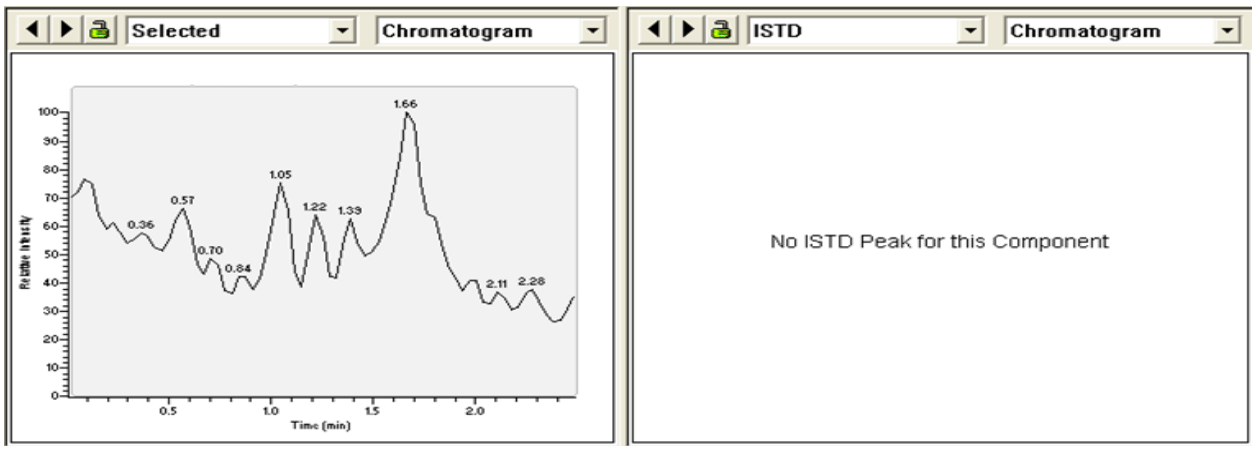
between an aqueous phase and organic phase (immiscible). This extraction method was useful for separating analyte from interferences present in biological matrices and different solutes depends on their degree of solubility in different solvents. During extraction of analyte and metabolite from plasma by using LLE with an extraction solvent t-BME, there is a significant improvement in peak shape, response and consistent results using any other extraction techniques (Solid Phase Extraction and Protein precipitation) and extraction solvents comparatively. After conducting many developmental trials, bioanalytical method was freezed with LLE technique to quantify AS and DHA from human plasma by using LC-MS/MS.

3.2 Method Validation

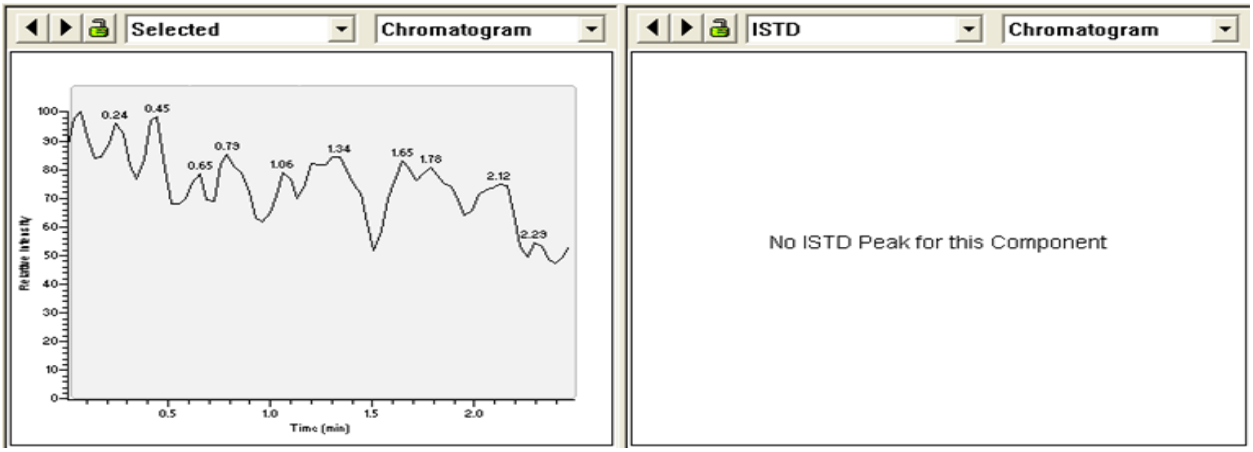
The method validation for AS and DHA in human plasma was performed by using LLE extraction method. As part of method validation selectivity, specificity, sensitivity, matrix effect, three precision and accuracy batches, linearity, recovery and stability (bench top, autoinjector, wet extract, freeze thaw stabilities) were evaluated according to the US Food and Drug Administration (FDA) and European medicines agency (EMEA) guidelines for the validation of bioanalytical method.[9,10] All the experiments conducted as part of method validation were accepted across all the stages. Results for each experiment were reported separately under experiments' subsection.

3.2.1 Selectivity, Specificity and Sensitivity

Selectivity was performed by selecting the six plasma lots. Analyte (LOQ standard) and ISTD were spiked individually into each plasma lot along with this blank samples were processed and analysed. Specificity of AS, DHA and ASD_3, $DHAD_4$ were evaluated in screened plasma lot for evaluating contribution of chemical interference from analyte, metabolite and internal standards vice versa. The interference at analyte, metabolite and internal standard RT should not be more than 20% and 5% respectively of LOQ standard response (sensitivity sample area taken for comparison). Sensitivity of the method was established by injecting LLOQ sample in six replicates. Sensitivity of the method should be within the 20% in precision and accuracy. After conducting experiment, results were verified for its acceptance and no interference was found in all plasma lots as well as at RT of analyte, metabolite and internal standard. Chromatograms represent selectivity, specificity and sensitivity curves are shown in Fig 8-10, and sensitivity results are shown in Table: 1.

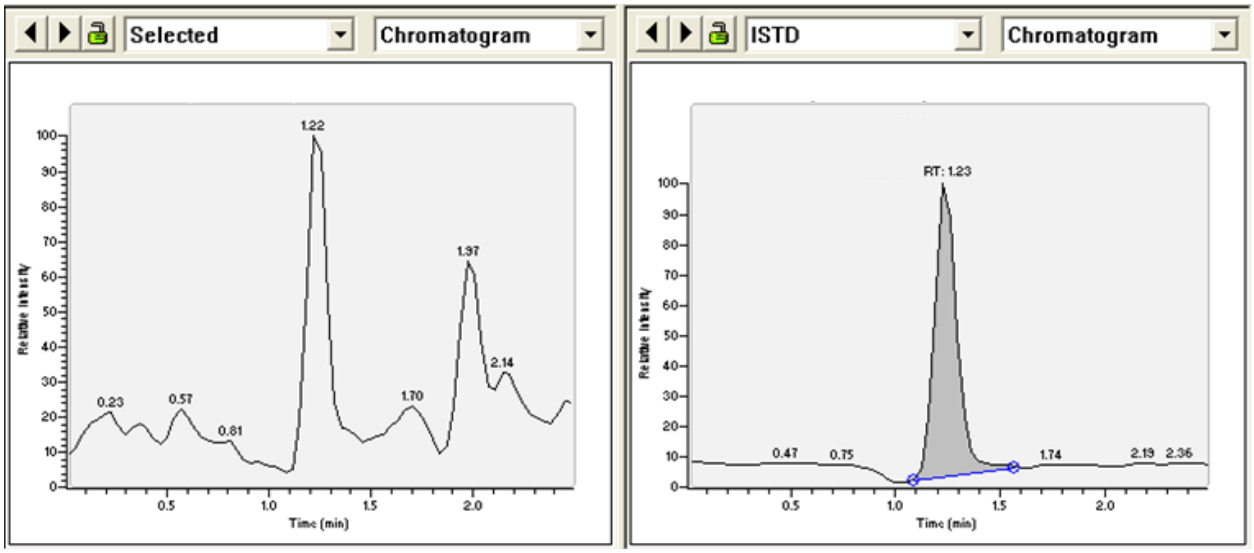


a) Artesnuate (AS).

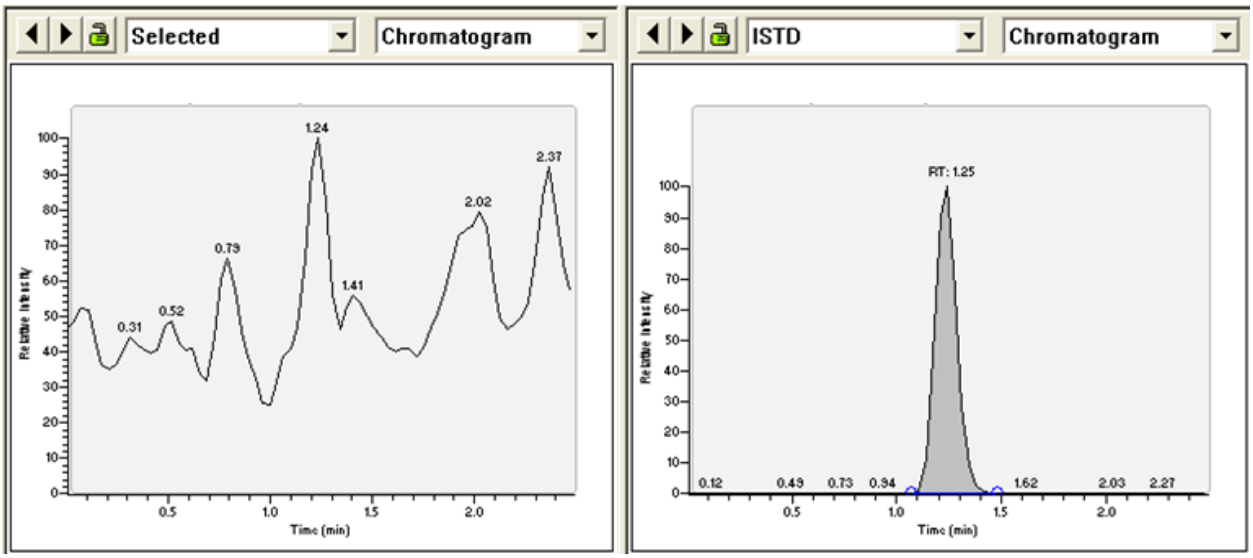


b) Dihydroartemisinin(DHA).

Fig-8: No interference from extracted blank both in Artesunate (AS) and Dihydroartemisinin (DHA).

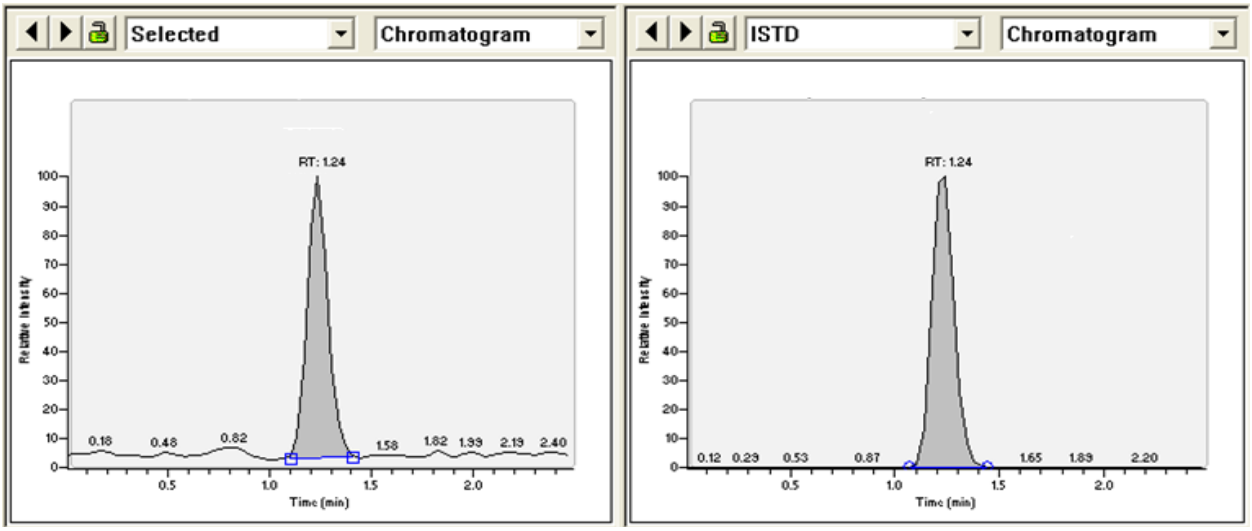


a) Artesunate (AS).

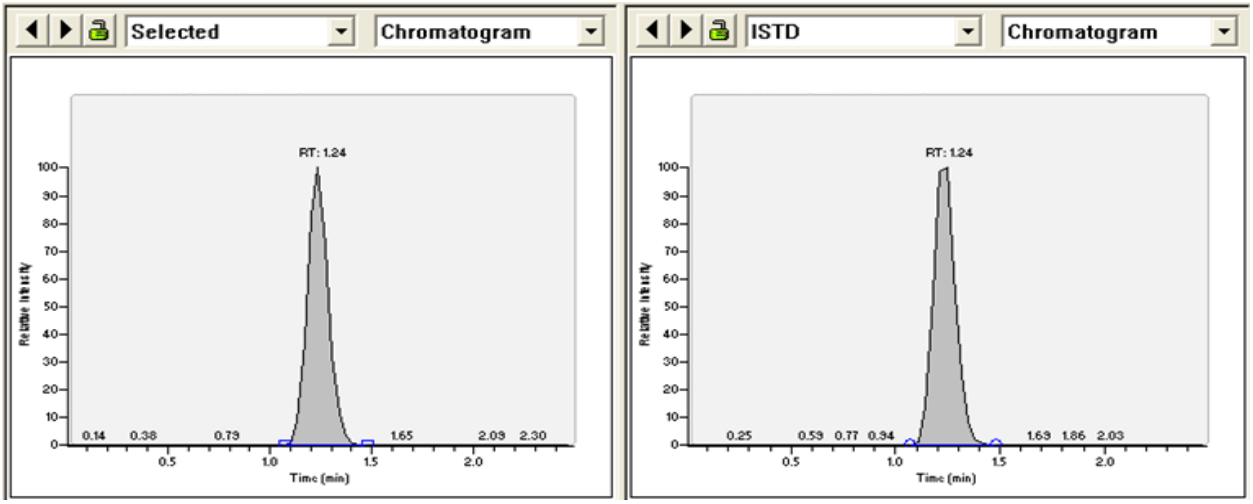


b) Dihydroartemisinin (DHA).

Fig-9: No interference from extracted IS at Artesunate (AS) and Dihydroartemisinin (DHA) RTs.



a) Artesunate (AS).



b) Dihydroartemisinin (DHA).

Fig-10: Extracted chromatogram of LLOQ with ISTD's.

Table-1: The analyte concentration at LLOQ in human plasma (n=6).

Analyte Name	Nominal Concentration (ng/mL)	Mean	Precision	Accuracy
AS	5.005	4.8747	8.9	97.4
DHA	10.026	11.4013	8.9	113.7

3.2.2 Matrix effect

To evaluate the effect of matrix counter ions present in the matrix on analyte, metabolite and internal standard of interest. An experiment was conducted by processing six blank samples at each QC level and spiked them with respective QC level of concentration. The aqueous samples were prepared based on the processing recovery based and compared with the post spiking of matrix samples. For analyte, metabolite and internal standard, the matrix factor was calculated for each lot of matrix, by calculating the ratio of matrix effect samples' area and aqueous samples area. The internal standard normalized factor calculated by using matrix factor of analyte, metabolite and internal standard. The %CV of such internal standard normalized factor from six lots were considered for evaluating the effect of matrix ions of analyte, metabolite of interest should not be more than 15%. After successful completion of the experiments, results were examined for matrix effect and results clearly indicated that, there is no matrix effect for analyte and metabolite quantification using current developed analytical method. The results for matrix effect are shown in Table: 2.

Table-2: Matrix effect in human plasma.

Nominal concentration (ng/mL)		Matrix effect % CV	
AS	DHA	AS	DHA
37501.950	75002.550	0.5	1.2
17501.910	35002.160	0.7	1.0
12.513	25.065	0.3	1.5

3.2.3 Precision and Accuracy and Recovery

Precision and accuracy describe the method closeness of repeated individual measures of analyte and closeness of the determined value obtained; those are expressed in CV and percentage respectively. Intra and inter day precision and accuracy of the analyte, metabolite were separately assessed by analyzing six replicates of QC's at LLOQ, LQC MQC and HQC level under standard calibration curve. At each QC level precision and accuracy within ± 15% where LLOQ level was ± 20% for both intra and inter day performance. The obtained results were calculated for precision, accuracy and outcomes were meeting the above stated criteria both in intra and inter-day acceptance. The results were shown in the Table. 3. To know the percentage of recovery of analyte, metabolite and internal standard from the spiked plasma; an experiment was performed by preparing the aqueous samples of each QC's level. The obtained areas of each QC level in aqueous samples were compared against the extracted samples areas in one of the precision and accuracy batches analytical results. The obtained global recovery for analyte and metabolite were found to be 82.5% and 85.3% respectively.

Table-3: Precision and Accuracy.

Analyte name	Nominal concentration (ng/mL)	Intra day			Inter day		
		Mean (n=6)	Precision	Accuracy	Mean (n=6)	Precision	Accuracy
AS	37501.950	6	7.6	96.6	6	5.5	99.6
	17501.910	6	7.8	106.9	6	5.2	101.3
	12.513	6	10.8	107.9	6	4.4	104.0
	5.007	6	11.8	105.0	6	3.7	100.9
DHA	75002.550	6	3.6	108.5	6	2.4	105.9
	35002.160	6	7.7	103.7	6	2.0	106.2
	25.065	6	9.6	103.7	6	5.2	98.8
	10.028	6	9.4	107.8	6	7.1	99.7

3.2.4 Standard Calibration Curve

The linearity was performed by spiking calibration standard samples at nine different concentration levels of analyte and metabolites' linearity ranges. The calibration curve linearity range includes both LLOQ and ULOQ are being lower and higher calibration standards. Calibration curves were typically described by the equation y = mx + c, where y corresponds to the peak-area ratio of AS and DHA to internal standard and x represents the plasma concentration of AS and DHA. The linearity of calibration curve was assessed by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/X^2$). The back calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration curve standard samples must be accepted. The results were reviewed for calibration curve acceptance and all the calibration curves performed to evaluate the precision and accuracy batches were accepted. Results of three calibration curves are shown in Table.4.

Table-4: Calibration curve.

Analyte	STD Level	Nominal Conc.	Back calculated Conc.			Precision	Accuracy
			Batch-I	Batch-II	Batch-III	%C.V	% Accuracy
AS	CC-1 STD	5.005	4.950	5.245	4.521	7.4	98.0
	CC-2 STD	10.012	10.120	12.493	9.946	13.1	108.4
	CC-3 STD	20.125	19.245	18.579	20.406	4.8	96.4
	CC-4 STD	50.007	45.258	53.178	56.231	11.0	103.1
	CC-5 STD	100.057	98.145	98.896	98.189	0.4	98.4
	CC-6 STD	200.092	205.452	199.912	188.369	4.4	98.9
	CC-7 STD	300.125	298.872	310.405	270.125	7.1	97.7
	CC-8 STD	400.034	380.489	389.186	409.850	3.8	98.3
	CC-9 STD	500.026	508.736	497.250	495.356	1.4	100.1
DHA	CC-1 STD	10.026	9.587	10.254	10.289	3.9	100.2
	CC-2 STD	20.063	19.368	20.568	22.145	6.7	103.1
	CC-3 STD	40.104	38.214	40.845	42.214	5.0	100.8
	CC-4 STD	100.042	104.860	108.657	112.369	3.5	108.6
	CC-5 STD	200.081	199.700	189.483	212.589	5.8	100.3
	CC-6 STD	400.126	409.125	401.200	420.864	2.4	102.6
	CC-7 STD	600.053	587.457	580.123	615.231	3.1	99.0
	CC-8 STD	800.114	851.487	789.820	815.452	3.8	102.4
	CC-9 STD	1000.034	983.145	1019.806	1040.502	2.9	101.4

3.2.5 Stabilities

Stability should be ensured for every step in the analytical method, meaning that the conditions applied to the stability tests, such as sample matrix, anticoagulant, container materials, storage and analytical conditions should be similar to those used for the actual study samples. All stabilities were evaluated at higher and lower QC level with six replicates under freshly prepared calibration curve standard and QC samples for comparison. The obtained concentrations are compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration. Different stability experiments were performed for AS and DHA autosampler stability (LC) at 5-10°C, bench top stability at room temperature, process sample stability at room temperature, dryextract stability at 2-8°C and freeze and thaw stability at -80°C and room temperature. Stability experiments were successfully meeting the specified acceptance criteria in precision, accuracy and percentage of stability. The results of AS and DHA for auto sample stability is 36 hr, bench top stability at room temperature is 9 hrs, four freeze and thaw cycles, processed sample stability is 6 hr 20 mins,

dry extract stability is 42 hrs. All stability results are shown in Table.5.

Table-5: Stabilities.

Experiment of Stability	Nominal concentration		Mean (n=6)		Precision		Accuracy	
	AS	DHA	AS	DHA	AS	DHA	AS	DHA
Auto sampler (LC)	37501.950	75002.550	6	6	5.3	3.4	94.6	98.1
	12.513	25.065	6	6	7.0	5.5	97.6	112.4
Bench top stability	37501.950	75002.550	6	6	3.5	3.0	98.6	107.5
	12.513	25.065	6	6	6.0	8.3	95.0	108.0
Processed sample	37501.950	75002.550	6	6	1.9	2.6	96.7	107.7
	12.513	25.065	6	6	4.2	4.2	93.1	109.6
Dry extract	37501.950	75002.550	6	6	3.4	2.7	97.9	110.0
	12.513	25.065	6	6	7.0	5.7	94.3	112.5
Freeze and thaw	37501.950	75002.550	6	6	5.4	2.1	101.2	109.7
	12.513	25.065	6	6	5.5	7.2	90.5	106.8

4. CONCLUSION

By observing above data we concluded that a selective LC-MS/MS method for the simultaneous determination of Artesunate and its metabolite Dihydroartemisinin in human plasma has been successfully developed and validated. A simple and inexpensive liquid-liquid extraction procedure and an isocratic chromatography condition using a reversed-phase column provided an assay well suited for real-time analysis. The method is found to be simple, specific, rugged and reproducible using a small sample volume 200 µL with short chromatographic run time (1.2 min). Hence the methods acceptable with respect to precision and sensitivity for quantification of both Artesunate and its metabolite Dihydroartemisininin human plasma and it can be applied for BA/BE studies.

5. ACKNOWLEDGMENT

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