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Original Article

SIMULTANEOUS DETERMINATION OF ARTESUNATE AND AMODIAQUINE IN HUMAN PLASMA USING LC-MS/MS AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

Objective: The objective of this research was to develop a simple, rapid and sensitive liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of artesunate and amodiaquine in human plasma.

Methods: An analytical method based on LC-MS/MS has been developed and validated for the simultaneous determination of artesunate and amodiaquine in human plasma. Isotope-labeled compounds are used as internal standards for the quantification of these drugs. Analytes were extracted from the plasma using solid phase extraction (SPE) technique and chromatographed on a C_8 column using an isocratic mobile phase composed of 0.1% ammonia solution and methanol (10:90, v/v). The mobile phase was pumped at a flow rate of 1.00 ml/min. A total of five analytical batches were generated for the calculation of intra-day and inter-day precision and accuracy during the entire course of validation.

Results: The assay exhibits excellent linearity in the concentration range of 3.07–305.29 ng/ml for artesunate and 0.30–30.01 ng/ml for amodiaquine. Intra-day and inter-day precision and accuracy results are well within the acceptance limits. All the stability experiments were conducted in plasma samples and in neat samples are complying with the recent US FDA and EMEA guidelines.

Conclusion: The proposed LC–MS/MS assay method is simple, rapid and sensitive enough for the simultaneous determination of artesunate and amodiaquine in human plasma. This method was successfully used to quantitate the *in-vivo* plasma concentrations obtained from a pharmacokinetic study and the results were validated by conducting incurred samples reanalysis (ISR).

Keywords: Artesunate and amodiaquine, Human plasma, LC–MS/MS, Method validation, Pharmacokinetics.

INTRODUCTION

Malaria is one of the most important infectious diseases in the world, causing hundreds of millions of illnesses and an estimated 1 million deaths each year. The disease is caused by *P. falciparum*. Many drugs are available for the treatment of uncomplicated malaria. The most important new class of antimalarial agents is the artemisinin. The World Health Organization (WHO) recently recommended the use of artemisinin-based combination therapy (ACT) for the treatment of uncomplicated P. falciparum malaria [1]. Artesunate (AS) is a water-soluble semi-synthetic derivative of artemisinin and can be given by injection [2]. Amodiaquine (AQ) is a 4-aminoquinoline compound similar to chloroquine, is also used as an antimalarial agent. A combination of AS and AQ is indicated for the treatment of uncomplicated cases of malaria due to *P. falciparum* strains which are susceptible to AQ as well as to AS [3]. AS/AQ combination for uncomplicated malaria is efficacious and safe in children also [4, 5]. This combination is drawing a certain degree of interest due to their half-life. AS is having a short half-life, whereas, AQ has a long half. The short half-life drug achieves substantial and rapid parasite killing while a high concentration of the long half-life drug kills off the remaining malaria parasites [6, 7].

As per the literature survey, many LC-MS/MS methods [8-17] have been reported for the determination of AS individually or along with its metabolite in a variety of biological samples. Similarly, one LC-MS/MS method [17] and one ion-pair liquid chromatography–tandem mass spectrometry method [18] has been reported for the determination of AQ along with its metabolite in biological samples. It is essential to develop a bioanalytical method suitable for simultaneous determination of AS and AQ in human plasma, a fixed dose combination (FDCs) is available in the market. These methods are helpful to examine the tolerability and safety profile of FDCs and/or for comparative bioavailability and bioequivalence studies. Also, analysis of FDCs in a single run reduces the experimental cost. To our knowledge, one high-performance liquid chromatography with electrochemical detection method [19] and one LC-MS/MS method [20] have been reported for the simultaneous determination of AS and AQ in human plasma. The conventional HPLC methods must sacrifice time, resolution or sensitivity [19].

Also, reported LC-MS/MS method is having limitations like gradient mobile phase composition, lack of specificity due to the improper characterization of more number of analytes in a single run, less sensitivity and longer chromatographic run time. In view of the above, the authors have attempted to develop a simple, specific, sensitive and rapid LC-MS/MS method for simultaneous determination of AS and AQ in human plasma using artesunate d4 (IS1) and amodiaquine d10 (IS2) as internal standards, respectively. The advantages of the proposed method over reported procedures in terms of specificity, selectivity, use of solid–phase extraction (SPE) technique without drying, evaporation and reconstitution steps, minimum usage of organic solvents, employed very low plasma volume (100 µl) and rapid with the chromatographic run time of 4 min which makes the method high throughput. Use of deuterated compounds as internal standards minimizes matrix effect related problems and variability in recovery between the analyte and the IS [21]. The method ensured the estimation of AS and AQ in real time samples collected from healthy male subjects up to 48 h of post dosing with desired accuracy and precision to support a pharmacokinetic study in healthy volunteers. The ISR results obtained demonstrated that the present method is highly reproducibility and suitable for the pharmacokinetic/bioequivalence studies in humans.

MATERIALS AND METHODS

Materials and reagents

The reference sample of AS (98.01%) and amodiaquine hydrochloride (91.56%), were obtained from Mangalam Drugs and

Organics Limited, India, whereas IS1 (100%) and IS2 (100%) were purchased from Vivan Life Sciences Private Limited, India. All the solvents used in the study are LC-MS grade. Methanol used as an organic modifier and purchased from J. T Baker (Phillipsburg, USA). Analytical grade ammonia and ammonium acetate were purchased from Merck, (Mumbai, India). Ultra-pure water was prepared at our laboratory using Milli Q water purification system procured from Millipore (Bangalore, India). Human blank plasma was obtained from Deccan's Pathological Labs, (Hyderabad, India).

LC–MS/MS instrument and conditions

An HPLC system (Shimadzu Corporations, Kyoto, Japan) coupled with AB Sciex API–4000 mass spectrometer (Foster City, CA, USA) was used for the study. A 15 µl aliquot of processed samples were injected into the analytical column (Kromasil C_8, 150 * 4.6 mm, 5 µm; Make: Akzo Noel) along with an isocratic mobile phase composed of 0.1% ammonia solution and methanol and (10:90, v/v). The mobile phase was pumped at a flow rate of 1.00 ml/min with splitter (50:50). The detailed mass spectrometric conditions and quantification parameters are listed in the table 1.

Preparation of calibration curve standards and quality control samples

Two separate stock solutions (1 mg/ml) of AS and AQ were prepared in HPLC grade methanol and used for the preparation of calibration curve (CC) standards and quality control (QC) samples. Further dilutions were made in a mixture of methanol and water (50:50, v/v; diluent). Likewise, IS1 and IS2 stock solutions (1 mg/ml) were prepared in methanol separately. A combined working solution for IS1 (1000 ng/ml) and IS2 (200 ng/ml) was also prepared in diluent. All the stock solutions were found to be stable for 21 d at 2–8 °C in refrigerator. An aliquot of 50 µl combined working standard dilution of AS and AQ was spiked into 950 µl of screened human plasma to obtain calibration standards and quality control samples. Calibrates were prepared at a concentrations of 3.07, 6.14, 15.34, 30.68, 61.36, 122.73, 183.17, 244.23 and 305.29 ng/ml for AS and 0.30, 0.60, 1.51, 3.02, 6.03, 12.06, 18.01, 24.01 and 30.01 ng/ml for AQ. The QC samples were prepared at five different concentration levels of 3.11 (lower limit of quantification, LLOQ), 9.21 (low quality control, LQC), 46.03 (middle quality control, MQC–1), 152.93 (MQC–2) and 255.74 (high quality control, HQC) ng/ml for AS and 0.31 (LLOQ), 0.91 (LQC), 4.53 (MQC–1), 15.04 (MQC–2) and 25.14 (HQC) ng/ml for AQ. All the prepared plasma samples were stored at–70±10 °C.

Sample extraction protocol

All frozen samples were thawed at room temperature and vortexed to ensure complete mixing of the contents. A 100 µl aliquot of plasma sample was pipetted into pre-labelled polypropylene tubes. Then 20 µl of internal standard working solution (a combined dilution of IS1 and IS2 at a concentration of 1000 ng/ml and 200 ng/ml, respectively) was added to it and vortexed for 10 s. To this, 200 µl of 100 mM ammonium acetate was added and vortex.

The sample mixture was loaded onto a Strata-X 33 µm polymeric sorbent (30 mg/1 ml) cartridge that was preconditioned with 1.0 ml of methanol followed by 1.0 ml of water and 1.0 ml of 100 mM ammonium acetate buffer. After applying the maximum pressure, the extraction cartridge was washed with 1.0 ml 100 mM ammonium acetate buffer followed by 2 ml of HPLC grade water (1.0 ml of each time). Analytes and the internal standards were eluted with 1.0 ml of mobile phase. Aliquot of 15 µL of the extract was injected into the chromatographic system.

Table 1: Tandem mass spectrometer main working parameters

Parameter	Analyte			
	AS	AQ	IS1	IS2
Mode of analysis	Positive	Positive	Positive	Positive
Ion transition, m/z	402.3/267.3	356.3/283.1	406.3/267.3	366.2/283.1
Source temperature, °C	550	550	550	550
Dwell time per transition, msec	200	200	200	200
Nebulizer gas, psi	50	50	50	50
TurboIon gas, psi	30	30	30	30
Curtain gas, psi	30	30	30	30
Collision gas, psi	4	4	4	4
Ion spray voltage, V	5500	5500	5500	5500
Entrance potential, V	10	10	10	10
Declustering potential, V	36	35	36	35
Collision energy, V	15	25	15	25
Collision cell exit potential, V	15	15	15	15
Resolution	Unit	Unit	Unit	Unit

Method validation

A complete and through validation was carried out as per US FDA [22] and EMEA guidelines [23]. The validation parameters tested are system suitability, carryover test, selectivity, specificity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, dilution integrity, stability, run size evaluation and method ruggedness.

Pharmacokinetic study protocol

A pharmacokinetic study was conducted in healthy male subjects (*n* = 7) under fasting condition using fixed dose combination (100/270 mg) of AS and AQ. The study was approved by the Independent Ethics Committee (Reg. No: ECR/70/Indt/AP2013) and conducted as per the GCP regulations. The subjects with an age group of 20–40 y and body–mass index (BMI) of ≥18.5 kg/m^2 and ≤24.9 kg/m^2, with body weight not less than 50 kg were selected for the study. All the subjects were fastened for 12 h before administration of the tablet formulation and kept in-house for 24 h during the study. Drinking water is restricted for one hour before and after dosing. Post-dosing, a standard meal is provided at specified intervals. Blood samples were collected at 0.083, 0.167, 0.25, 0.333, 0.5, 0.667, 0.833, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 16, 24, 36 and 48 h of post dose in K2 EDTA vacutainer (5 ml) collection tubes (BD, Franklin, NJ, USA). A predose sample was also collected from each subject to check the possible interferences. The blood samples were centrifuged at 3200 rpm for 10 min and the plasma was collected and stored at–70±10 °C till their use. The pharmacokinetic parameters of AS and AQ are calculated by using Win Nonlin software (Version 5.2). Non–compartmental model was employed for the preset study. The study data was validated through ISR. For ISR, a total of 14 samples (two samples from each subject) were selected for each analyte near to C_{max} and the elimination phase. The percent change deviation between initial and ISR value allowed is±20% [24, 25].

RESULTS AND DISCUSSION

Mass spectrometry

The purpose of the study was to develop and validate a LC-MS/MS method for the simultaneous determination AS and AQ in human

plasma suitable for the pharmacokinetic and bioavailability or bioequivalence. Analysis of multiple analytes in a single run considerably reduces the researchers' time and experimental cost. Hence, rapid, specific and sensitive methods like liquid chromatography coupled with mass spectrometry are required to analyze the drug candidates in biological samples. Mass spectrometric conditions were optimized in positive and negative ionization modes using tuning solution. The high-intensity response was obtained in positive mode than the negative mode for AQ and the IS2. But the intensity response obtained for the AS and IS1 was very low and not enough to quantify. Hence, ammonia solution was added to a stock solution of AS and IS1 (0.3 ml in 10 ml) to produce ammonium adduct $(M+NH_4)^+$ and the same was detected. Compound parameters like declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were optimized to obtain most intense and consistent production Q3 MS spectra of analytes and the internal standards. The source parameters like nebulizer gas (GS1), auxiliary gas (GS2), collision gas (CAD), temperature and ion spray voltage were optimized to obtain an adequate and reproducible response. Detection of the ions was carried out in the multiple reaction monitoring (MRM) mode by monitoring the transition pairs of m/z 402.3 $(M+NH_4)^+$ precursor ion to the m/z 267.3 for AS, m/z 356.3 precursor ion to the m/z 283.1 for AQ, m/z 406.3 $(M+NH_4)^+$precursor ion to the m/z 267.3 for IS1 and m/z 366.2 precursor ion to the m/z 283.1 production for the IS2. The dwell time for each transition was set at 200 ms. multiple reactions monitoring (MRM) was chosen for the present assay development as this technique provides inherent selectivity and sensitivity for pharmacokinetic studies [26].

Chromatographic conditions

The critical chromatographic conditions such as mobile phase composition, flow rate, column type and injection volume were carefully monitored to obtain the good resolution from the endogenous components and to give better sensitivity with short run time. The composition of the mobile phase was elevated with acetonitrile and methanol by varying its proportion with volatile buffers like ammonium formate, ammonium acetate, as well as basic additives like ammonia solution in varying strength. Finally, the promising results were archived with an isocratic mobile phase composed of 0.1% ammonia solution in water and methanol (10:90, v/v) as gave symmetric peak shape, better separation and best sensitivity for the analytes. Among the various chromatographic columns (Kromasil C_8; Hypurity advance; Zorbax SB C_{18}; Kromasil 100–5C_{18}; Ace 3 C_{18}; Alltima HP C_{18}; Zorbax XDB–phenyl; Discovery HS C_{18}) tested for their suitability Kromasil C_8, 150 × 4.6 mm, 5 µm column gave good peak shape and response even at lowest concentration level for both the analytes. The mobile phase flow rate was set at 1.0 ml/min allowing a run time of 4 min.

Optimization of extraction procedure

The reported procedures have employed protein precipitation (PP) [12, 18, 20], liquid-liquid extraction (LLE) [8, 13, 15] and SPE [9, 10, 16, 17, 19] with drying evaporation and reconstitution steps to extract AS and AQ from biological samples. PP is most likely to cause ion suppression, since this method fails to remove sufficiently endogenous compounds such as lipids, phospholipids, fatty acids, etc. Similarly, LLE requires more organic solvent for extraction causes environmental hazards due to inhalation of the non-polar organic solvents. SPE is the most popular sample preparation technique due to following advantages: high recovery, effective sample preparation, required less organic solvent compared to LLE, ease of operation and a greater possibility of automation. Also, SPE technique gives clear extracts than the PP and LLE hence the influence on sensitivity is significantly less. Hence, SPE was tested with Bond Elut Plexa, Oasis HLB, and Orpheus C18 extraction cartridges with/without acidic buffer addition to obtaining the clean sample without any interference. Among the various extraction cartridges tested, promising results were obtained with Strata–X 33 µm polymeric sorbent cartridge gave superior recovery for the analytes compared with PP and LLE. Use of ammonium acetate buffer (100 mM) as an extraction helped in achieving reproducible and highest recoveries for both the analytes. Also, ammonium acetate buffer used as a washing solvent during SPE procedure to eliminate the possible interference. Use of mobile phase as an eluting solvent helped in achieving reproducible and quantitative recovery for the analyte devoid of drying and reconstitution steps.

Internal standard selection

Internal standard selection is an important aspect to achieve acceptable method performance, especially with LC-MS/MS, where matrix effects can lead to poor analytical results [27]. Deuterated standards of the analyte as an internal standard are helpful to obtain increased precision and accuracy results in bioanalysis where significant matrix effect is possible. Also, limit the recovery variation between the analyte and internal standard. Hence, deuterated standards of AS and AQ such as artesunate d4 and amodiaquine d10 are used as internal standards, respectively and were found to be best for the present purpose.

System suitability

System suitability test was performed before the start of everyday analysis. A mixture of a neat sample containing analytes at middle QC concentration and internals standards at working concentration were injected for the system suitability experiment. The precision of system suitability test is within the acceptance limits during the entire method validation. The precision (% CV) for system suitability test was found to be less than 1% for retention time and 1.3 % for area ratio of AQ and AS.

Selectivity and chromatography

Selectivity of the proposed method was evaluated by using 6 control human plasma lots (4 were normal and one lipemic and one haemolyzed). Each lot was analyzed as blanks and after spiking the plasma with the analyte at the LLOQ for any interference. The degree of interference by endogenous plasma constituents with the analytes and IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Figs. 1A & 2A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug–free plasma at the retention time of the analytes. fig. 1B and 2B depicts an LLOQ sample of AS and AQ along with the respective internal standards. Similarly, no interference was observed from commonly used medications such as acetaminophen, diphenhydramine, pantoprazole, nicotine, ibuprofen, caffeine and pseudoephedrine. No effects of cross-talk were observed. Fig. 3 and 4 depicts a representative chromatogram of AQ and AS resulting from the analysis of subject blank plasma sample along with the respective IS and 0.333 h subject plasma sample after the single oral dose of a AQ (100 mg) and AS (270 mg) tablet.

Sensitivity

Analyte sensitivity (lowest limit of reliable quantification; LLOQ) was determined at the concentration of 3.07 ng/ml for AS and 0.30 ng/ml for AQ. At this concentration, the precision and accuracy results of AS were found to be 3.12% and 95.09%. Similarly, the precision and accuracy results of AQ were found to 3.24% and 102.48%. The signal–to–noise ratio (S/N) was measured at LLOQ concentration and found to be ≥10 for both the analytes.

Matrix effect

Sample preparation with SPE procedure found free from significant matrix effect. This experiment was done with the aim to check the effect of six lots of plasma on the back calculated value of QC's nominal concentration. The precision and accuracy for AS at LQC concentration were found to be 1.62% and 94.88%, and at HQC level they were 3.09% and 99.01%, respectively. Similarly, the precision and accuracy for AQ at LQC concentration were found to be 3.50% and 92.14%, and at HQC level they were 3.85% and 101.49%, respectively. No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high-quality control concentrations.

Calibration curve and linearity

Nine–point calibration curve was found to be linear over the concentration range of 3.07–305.29 ng/ml for AS and 0.30–30.01 ng/ml for AQ. 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–response detector relationship for both the analytes in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥ 0.99 for both the analytes.

Precision and accuracy

Intra-day precision and accuracy results were calculate using two different batches analyzed on a single day, whereas inter-day results were calculated using five different batches analyzed on a three successive day. The overall results are summarized in table 2.

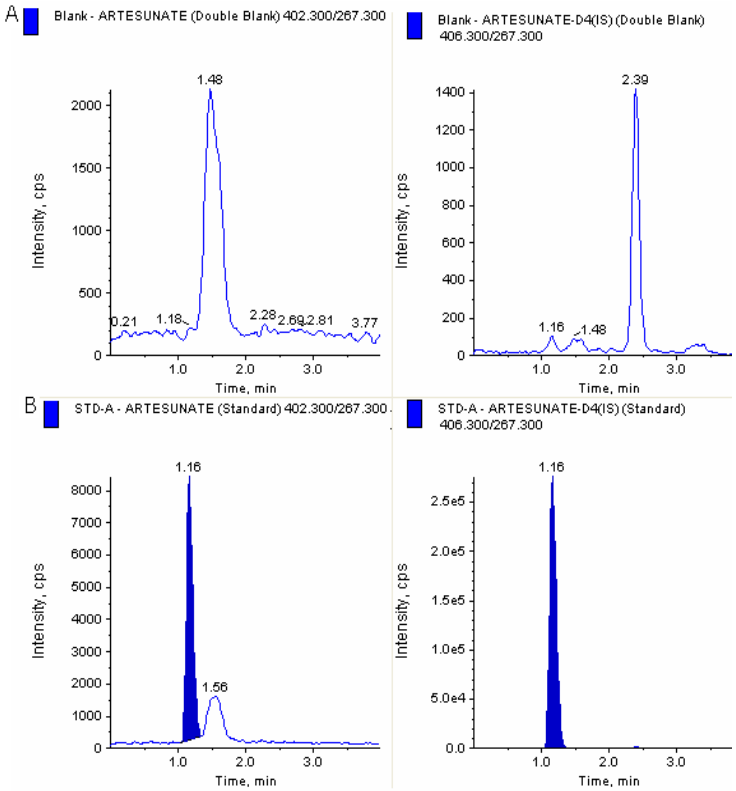

Fig. 1: Typical MRM chromatograms of AS (left panel) and the IS1 (right panel) in human blank plasma (A) and a LLOQ sample along with IS1 (B)

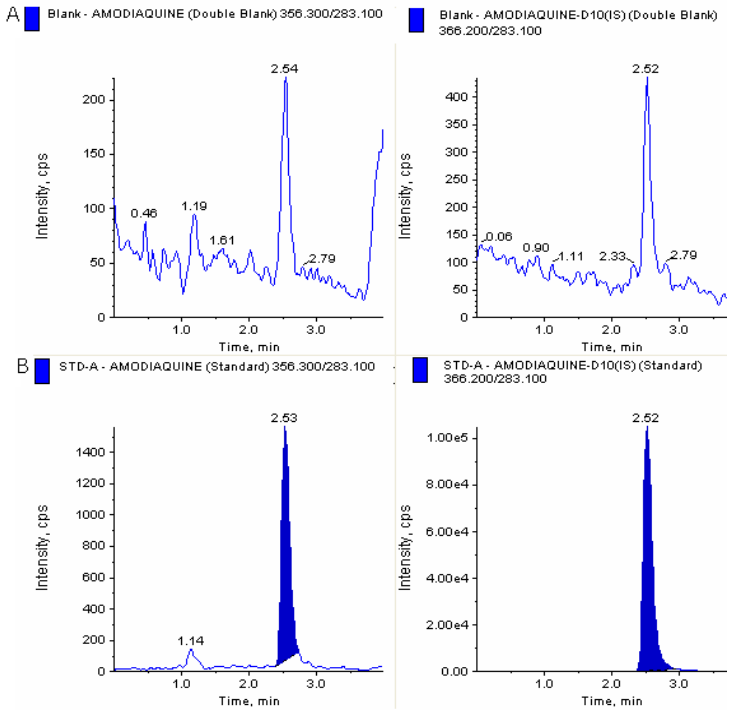

Fig. 2: Typical MRM chromatograms of AQ (left panel) and the IS2 (right panel) in human blank plasma (A) and a LLOQ sample along with IS2 (B)

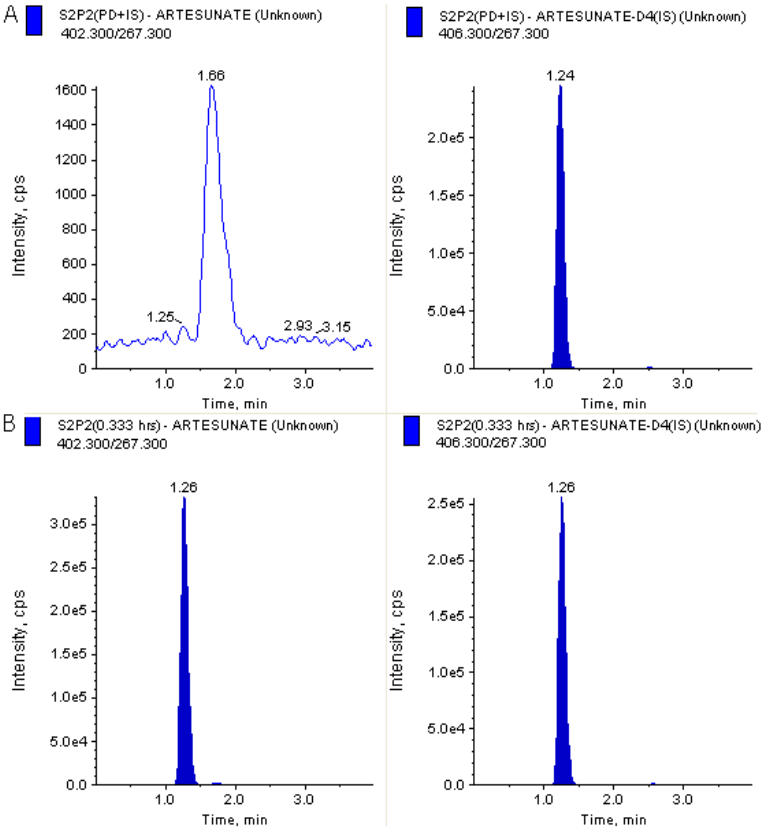

Fig. 3: Typical MRM chromatograms of a 0.333 h plasma sample (294.88 ng/ml) showing AS peak along with IS1 obtained following oral administration of 100 mg AS tablet to a healthy volunteer

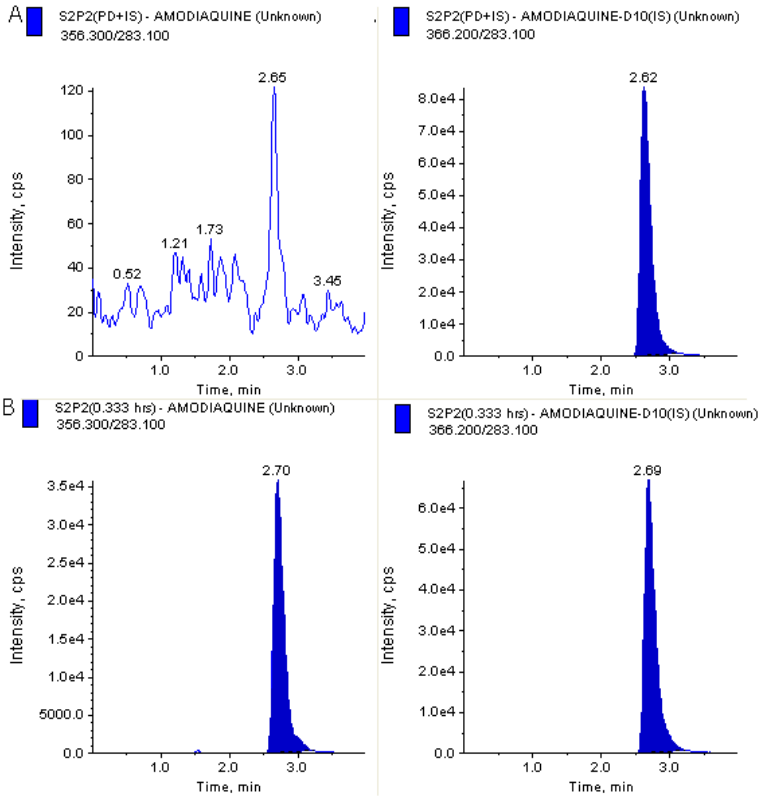

Fig. 4: Typical MRM chromatograms of a 0.333 h plasma sample (10.96 ng/ml) showing AQ peak along with IS2 obtained following oral administration of 270 mg AS tablet to a healthy volunteer

Table 2: Intra–day and inter–day precision and accuracy data for AS and AQ

QC		Intra–day precision and accuracy (*n*=12; 6 from each batch)			Inter–day precision and accuracy (*n*=30; 6 from each batch)		
Analytes	**Concentration spiked (ng/ml)**	**Concentration found (mean; ng/ml)**	**Precision (%)**	**Accuracy (%)**	**Concentration found (mean; ng/ml)**	**Precision (%)**	**Accuracy (%)**
AS	3.11	3.20±0.19	5.94	102.69	3.25±0.22	6.88	104.37
	9.21	9.91±0.44	4.48	107.65	9.66±0.59	6.12	104.94
	46.03	48.75±3.01	6.17	105.90	46.59±2.81	6.04	101.20
	152.93	154.66±7.04	4.55	101.13	148.38±7.80	5.26	97.03
	255.74	254.32±8.03	3.16	99.45	243.11±12.52	5.15	95.06
AQ	0.31	0.32±0.02	6.33	103.57	0.31±0.02	8.01	101.48
	0.91	0.82±0.02	2.64	90.99	0.83±0.03	3.15	92.05
	4.53	4.08±0.13	3.27	90.10	4.14±0.11	2.58	91.38
	15.04	13.66±0.37	2.67	90.86	13.52±0.27	2.03	89.95
	25.14	23.13±0.51	2.19	92.01	23.10±0.36	1.55	91.87

Extrication efficiency

The recoveries of analytes and the internal standards with SPE procedure were good and reproducible. Recovery of the analytes was determined at three concentration levels i.e. LQC, MQC2 and HQC. The overall mean recovery (with the precision range) of AS was found to be 92.20 ± 1.05% (4.21–7.69%), whereas for AQ was 88.94 ± 4.44% (3.57–6.16%). Similarly, the recovery (with the precision range) of the IS1 and IS2 were 92.57% (5.06–7.83%) and 85.21% (5.24–8.67%), respectively.

Dilution integrity

Dilution integrity experiment was conducted to extend the upper limit of quantification suitable for higher dose of AS and AQ. Dilution integrity test was performed using a concentration of 508.81 ng/ml for AS and 50.02 ng/ml for AQ. The precision and accuracy for AS at 1/2 dilution were found to be 1.39% and 97.44%, and at 1/4 dilution they were 2.00% and 101.39%, respectively. Similarly, the precision and accuracy for AQ at LQC concentration were found to be 5.10% and 102.85%, and at HQC level they were 5.91% and 105.43%, respectively.

Stability studies

Stability of analytes in human plasma samples and stability of processed samples was evaluated under different conditions. A wide range stability experiments namely auto–sampler stability (75 h), repeated freeze–thaw cycles (4 cycles), bench top stability (13 h), reinjection stability (52 h), wet extract stability (72 h at 2–8 °C) and long–term stability at–20 °C &–70 °C for 87 d had been performed throughout validation. The mean % nominal values were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels and the precision (% CV) values were within 15% (table 3). All the stability study results were well within the specified limits over the total validation.

Table 3: Stability samples result for AS and AQ (*n*=6)

Analyte	Stability test	QC (spiked concentration (ng/ml)	Mean ± SD (ng/ml)	Accuracy/ Stability (%)	Precision (%)
AS	Process[a]	9.21	8.48 ± 0.89	92.16	10.48
		255.74	251.83± 9.75	98.47	3.87
	Process[b]	9.21	9.22 ± 0.66	100.11	7.11
		255.74	234.71 ± 3.16	91.78	1.35
	Bench top[c]	9.21	10.01 ± 0.36	108.70	3.60
		255.74	253.95 ± 8.47	99.30	3.34
	FT[d]	9.21	9.14 ± 0.43	99.23	4.68
		255.74	235.61 ± 8.07	92.13	3.43
	Reinjection[e]	9.21	10.01 ± 0.36	108.76	3.60
		255.74	256.37 ± 2.82	100.25	1.10
	Long–term[f]	9.21	8.78 ± 0.50	95.37	5.66
		255.74	246.15 ± 9.98	96.25	4.05
AQ	Process[a]	0.91	0.93 ± 0.02	102.30	2.31
		25.14	25.23 ± 0.12	100.34	0.47
	Process[b]	0.91	0.80 ± 0.02	88.20	2.63
		25.14	22.78 ± 0.26	90.59	1.15
	Bench top[c]	0.91	0.82 ± 0.03	90.96	3.40
		25.14	22.73 ± 0.34	90.38	1.49
	FT[d]	0.91	0.82 ± 0.02	90.26	2.10
		25.14	22.70 ± 0.36	90.30	1.60
	Reinjection[e]	0.91	0.85 ± 0.04	93.66	4.19
		25.14	25.60 ± 1.14	101.82	4.47
	Long–term[f]	0.91	0.88 ± 0.07	97.46	8.11
		25.14	24.49 ± 0.55	97.39	2.25

[a] after 75 h in autosampler at 10°C; [b] after 72 h in refrigerator at 2–8°C; [c] after 13 h at room temperature; [d] after 4 freeze and thaw cycles; [e] after 52 h of Reinjection; [f] at–70°C for 87 d Stock solutions of AS, AQ, IS1 and IS2 were found to be stable for 28 d in refrigerator at 2–8 °C. The percentage stability (with the precision range) of AS, AQ, IS1 and IS2 was 99.64% (1.02–3.39%), 102.79% (0.96–1.55%), 99.34% (2.25–2.86%) and 97.92% (1.99–4.65%), respectively.

Long run evaluation

Long run evaluation experiment was carried out to assess the total number of the samples can be analyzed in a single run. A batch size of 195 samples containing 40 sets each of LQC, MQC1, MQC2 and HQC (samples stored at–70 °C) and 24 freshly spikes QC samples (6 sets at each level) were analyzed for the long run evaluation. These quality control samples were quantified by using a freshly spiked

calibration curve standards. For AS, 160 QC's out of 160 QC's of stored QC's and 24 QC's out of 24 QC's of freshly prepared QCs were within 15% of their respective nominal (theoretical) values. Similarly, 149 QC's out of 160 QC's of stored QC's and 23 QC's out of 24 QC's of freshly prepared QCs for AQ were within 15% of their respective nominal (theoretical) values.

Ruggedness

The ruggedness of the method was established by analyzing one precision and accuracy batch on the different instrument of the same make. Also, the precision and accuracy batch was processed by the analyst who was not involved in the study. The precision (%CV) and accuracy values for AS were ranged from 1.57–10.68% and 93.78–103.59%, respectively. The precision (%CV) and accuracy values for AQ were ranged from 1.14–7.12% and 88.59–109.04%, respectively.

Pharmacokinetic study results

The validated method was then tested for its applicability to a pharmacokinetic study in 7 healthy male subjects. The obtained pharmacokinetic results were listed in table 4. The mean plasma concentration *vs* time profile of AS and AQ are displayed in fig. 5 (presented up to 10 h in order to depict the plot with better clarity).

Table 4: Pharmacokinetic parameters of AS and AQ (n=6, mean±SD)

Parameter	AS	AQ
C_{max} (ng/ml)	222.10±48.61	11.90±2.25
t_{max} (h)	0.26±0.06	0.83±0.33
AUC_{0-t} (ng h/ml)	88.25±30.24	70.05±3.68
AUC_{0-inf} (ng h/ml)	92.21±30.88	79.56±6.23
$t_{1/2}$ (h)	0.48±0.21	15.44±5.80
Kel (h^{-1})	1.71 ± 0.74	0.05 ± 0.02

Incurred sample reanalysis

Currently, US FDA introduced the importance of incurred sample reanalysis (ISR) to authenticate the study data. A total of 14 samples for each analyte were evaluated for ISR. The differences in concentrations between the ISR and the initial values for all the tested samples were less than 15% (table 5), indicating good reproducibility of the present method.

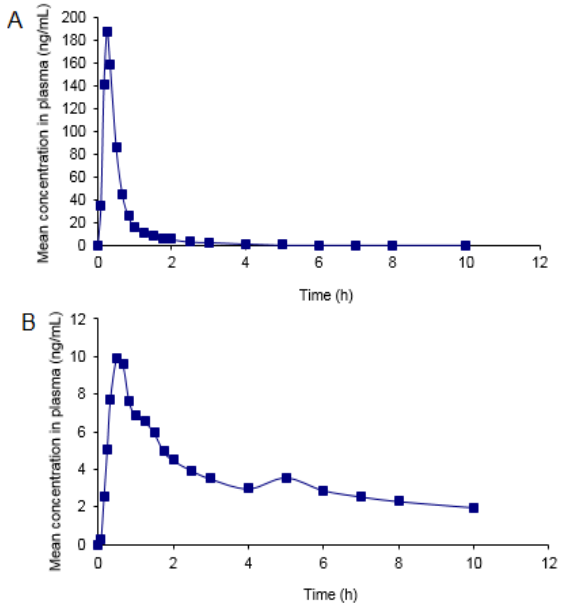

Fig. 5: Mean plasma concentration–time profile of AS (A) and AQ (B), in human plasma following oral dosing of AS (100 mg) and AQ (270 mg) tablet to healthy volunteers (n = 7)

Table 5: Incurred samples re–analysis data of AS and AQ

Subject no.	AS				AQ			
	Sampling point (h)	Initial conc. (ng/ml)	Re–assay conc. (ng/ml)	Difference[a] (%)	Sampling point (h)	Initial conc. (ng/ml)	Re–assay conc. (ng/ml)	Difference[a] (%)
1	0.167	150.78	141.21	6.55	0.667	14.216	13.621	4.27
1	2	10.50	9.62	8.66	24	1.096	0.991	10.06
2	0.25	177.72	160.29	10.32	0.333	10.964	11.970	-8.77
2	1.75	13.51	13.92	-3.01	16	1.003	1.124	-11.38
3	0.25	194.76	175.70	10.29	0.833	6.404	6.838	-6.55
3	0.833	11.60	10.90	6.21	12	1.299	1.205	7.51
4	0.167	133.40	139.88	-4.74	0.5	8.438	8.134	3.67
4	1	10.69	10.90	-1.94	24	1.012	0.991	2.10
5	0.167	202.89	199.23	1.82	0.333	5.255	5.358	-1.94
5	1	11.86	12.32	-3.82	10	1.524	1.644	-7.58
6	0.333	193.59	214.31	-10.16	0.833	8.335	8.228	1.29
6	1.5	11.83	10.59	11.08	20	1.045	1.120	-6.93
7	0.25	106.48	110.22	-3.46	0.667	12.110	11.964	1.21
7	1.5	12.90	11.36	12.64	24	1.546	1.507	2.55

[a] Expressed as [(initial conc. –re–assay conc.)/average]×100%.

CONCLUSION

In ultimate analysis, it can be vouchsafed that, we have developed and validated a sensitive, selective and rapid LC–MS/MS method in MRM mode for the simultaneous determination of AS and AQ in human plasma. The method utilizes deuterated standards as internal standards for quantification. Hence, possible matrix effect related problems are eliminated effectively. Additionally, sample preparation using SPE method with direct injection (no drying and reconstitution) gave consistent and reproducible recoveries for the analytes from the human plasma. The stability of AS and AQ in plasma and in aqueous samples under different conditions has been extensively evaluated and the results met the acceptance criteria as per recent FDA guidelines. The method showed suitability for clinical studies in humans. In addition, assay reproducibility is effectively proved by incurred sample reanalysis.

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ABBREVIATION

LC-MS/MS, Liquid Chromatography/Tandem Mass Spectrometry; MRM, Multiple Reaction–Monitoring Mode; US FDA, United States

Food and Drug Administration; IOP, Intraocular Pressure; HPLC, High Performance Liquid Chromatography; PP, protein precipitation; IS, Internal Standard; SPE, Solid–Phase Extraction; LLE, Liquid-liquid extraction; CC, Calibration Curve; QC, Quality Control; DP, Declustering Potential; CE, Collision Energy; EP, Entrance Potential; CXP, Collision Cell Exit Potential; LLOQ QC, Lower Limit of Quantitation Quality Control; LQC, Low Quality Control; MQC, Medium Quality Control; HQC, High Quality Control; EDTA, Ethylenediaminetetraacetic acid; ER, Extended Release; ISR, Incurred Sample Reanalysis; ESI, Electro Spray Ionisation; ULOQ, Upper Limit of Quantitation.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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