JOURNAL OF ADVANCEMENT IN MEDICAL AND LIFE SCIENCES

Journal homepage: http://scienceq.org/Journals/JALS.php

Research Article Open Access

# Bioanalytical Method Development and Validation for the Estimation of Lumefantrine and Desbutyl Lumefantrine in Human Plasma by LC-MS/MS

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Received: October 29, 2015, Accepted: December 15, 2015, Published: December 15, 2015.

## ABSTRACT

A liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) method for the simultaneous quantitation of lumefantrine (LF) and its active metabolite, desbutyl lumefantrine (DBL) metabolite in human plasma is developed and validated. The chromatographic separation was carried out on Hypersil Gold C18 (50 × 4.6 mm, 5 µm) using 5.0 mM ammonium formate (pH 3.0 adjusted with formic acid): acetonitrile (10:90, *v/v*) as the mobile phase. For sample preparation, solid phase extraction was carried out using artemisinin as the internal standard (IS) from 100 µL human plasma. Quantitation of analytes was done by tandem mass spectrometer using electrospray ionization in the positive mode. The precursor to product ion transitions monitored were *m/z* 530.5→ 512.1, *m/z* 472.7→512.1 and *m/z* 300.2 →219.5 for LF, DBL and IS respectively. The calibration curve was linear over the concentration range of 5.0–5000 ng/mL for both the analytes with a correlation coefficient ($r^2$) ≥ 0.9992. The intra-day and inter-day assay precision ranged from 1.77 to 7.22 % and 0.96 to 3.90 % for LF and 2.36 to 8.12 % and 0.57 to 4.64 % for DBL respectively. Similarly, the intra-day and inter-day assay accuracy was between 98.3–101.2 % and 98.8–102.1 % for LF and 97.5–101.6 % and 98.2–101.7 % for DBL respectively. The mean extraction recovery of LF and DBL was 98.5 % and 98.4 % across four quality control levels. Stability study in plasma was evaluated under different conditions like bench top, auto sampler, freeze-thaw and long term. The application of this assay was demonstrated through a bioequivalence with 12 healthy subjects using 20/120 mg artemether/lumefantrine tablet formulation.

**Keyword:** *Lumefantrine, desbutyl lumefantrine, LC-MS/MS, solid phase extraction, bioequivalence*

## INTRODUCTION:

Malaria is a foremost cause of morbidity and mortality in the developing regions of the world and remains the leading health issue in endemic regions [1]. Lumefantrine (LF) is a 2,4,7,9-substituited fluorene (2,3-benzindene) derivative and is commercially available in combination with artemether as Co-artemether (Riamet®). This artemisinin based combination therapy (ACT) has proved to be highly effective for the treatment of uncomplicated *falciparum* malaria in children and adults [2]. In addition, LF shows marked blood schizontocidal activity against wide range of *Plasmodium* including *Plasmodium falciparum*. Biochemical studies have revealed that the anti-malarial action of LF involves lysosomal trapping of the drug in the food vacuole of the intra-erthrocytic parasite, followed by binding to haem. Thus, the polymerization of haem is prevented and thereby inhibiting the detoxification of haem [3]. LF is mainly metabolized (to a low extent) by liver microsomal cytochrome P450 isoenzyme 3A4 into its putative metabolite desbutyl lumefantrine (DBL) [4]. LF is a highly lipophilic in nature and its human serum protein binding is about 99 %. The maximum plasma concentration is attained within 6-7 h. LF has a much longer elimination half-life (several days) and is associated with a low recrudescence rate, but has a slower onset of action.The elimination half-life of DBL is longer than LF [2, 4-5].

Literature presents several methods to quantify LF as a single analyte [6-12] in different biological matrices like rat plasma [6], mouse whole blood [7], dried blood spots [8, 9], human plasma [10-12]. Simultaneous analysis of LF together with DBL in human plasma [13-18] and with other anti-malarial or anti-retroviral drugs [19-21] is also reported. In these methods analytical techniques like HPLC with UV/fluorescence [8, 9, 11, 13, 15, 17, 20, 21] and mass spectrometric detection [6, 7, 10, 12, 14, 16, 18, 19] have been employed. The salient features of methods for the simultaneous quantification of LF and DBL in human plasma are summarized in **Table 1**.

In the present work we report an improved LC-MS/MS method for determination of LF and DBL in human plasma with respect to the sensitivity, analysis time and plasma sample volume over existing methods. The method was applied to a bioequivalence study in 12 healthy subjects using 20/120 mg artemether/lumefantrine tablet formulation.

Table 1 Comparative assessment of chromatographic methods developed for lumefantrine and desbutyl lumefantrine in human plasma

| Sr. No. | Technique; linear range (ng/mL) | Extraction procedure; plasma volume (µL) | Retention time LF/DBL; run time (min) | Application | Ref. |
|---|---|---|---|---|---|
| 1 | HPLC-UV; 24-20,000 for LF and 21-1010 for DBL | SPE; 250 | 16.5/10.0; 25.0 | Analysis of LF from plasma samples obtained from clinical trials of artemether-lumefantrine | 13 |
| 2 | HPLC-UV; 12-12000 for both the analytes | LLE; 200 | 7.25/4.86; 10.0 | Measurement of LF and DBL in plasma samples of patients | 15 |
| 3 | HPLC-UV; 10-12000 for both the analytes | LLE; 200 | 2.7 and 6.1; 10.0 | The method was applied for determination of LF and DBL concentrations in a pharmacokinetic food-drug interaction study | 17 |
| 4 | LC-MS/MS; 4-4000 | PP; 200 | 15.0/13.1; 17.0 | Simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma | 14 |
| 5 | LC-MS/MS; 2.0-2000 for both analytes | PP followed by SPE; 100 | 8.28/7.20; 9.0 | The method was applied to determine LF and DBL in 24 patients | 16 |
| 6 | LC-MS/MS; 21-529 for LF and 1.9-47 for DBL | PP; 100 | 1.7/1.5; 2.2 | The method was applied to determine plasma LF and DBL concentration in children under 5 years of age | 18 |
| 7 | LC-MS/MS; 5.0-5000 for both the analytes | SPE; 100 | 1.81/0.90; 2.50 | Bioequivalence study with 20/120 mg tablet formulations of artemether/lumefantrine in 12 healthy volunteers | PM |

LF: lumefantrine; DBL: desbutyl lumefantrine; PP: protein precipitation; LLE: liquid-liquid extraction; SPE: solid phase extraction; PM: present method

## EXPERIMENTAL

### Chemicals and materials

Reference standards of lumefantrine (LF, 98.14%), desbutyl lumefantrine (DBL, 99.52%) and artemisinin (IS) (ARM, 99.33%) were procured from Clearsynth Labs (P) Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, (S.A.de C.V. Mexico). Guaranteed reagent grade formic acid and ammonium formate were obtained from Merck Specialties Pvt. Ltd., (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Oasis HLB extraction cartridges (30 mg/1 mL) were purchased from Waters (Bangalore, India). Blank human plasma was procured from Supratech Micropath (Ahmedabad, India) and was stored at -20 °C until use.

### Optimized liquid chromatography and mass spectrometry conditions

A Shimadzu HPLC system (Kyoto, Japan) equipped with LC-20AD pump was used for the separation of the analytes on a Thermo Scientific Hypersil Gold C18 (50 × 4.6 mm, 5 µm) column, maintained at 40 °C in a column oven. The mobile phase consisted of 5.0 mM ammonium formate, (pH 3.0 adjusted with formic acid): acetonitrile (10:90, *v/v*) and was delivered at the flow rate of 1.0 mL/min. Detection of analytes and IS was performed on a triple quadrupole mass spectrometer, API-4000 equipped withTurbo Ion spray®, manufactured by MDS SCIEX (Toronto, Ont., Canada) and operating in the positive ionization mode. For the analytes and IS the source dependant parameters maintained were Gas 1 (Nebulizer gas): 15 psi, Gas 2 (heater gas): 10 psi, ion spray voltage (ISV): 5000 V, turbo heater temperature: 400 °C, entrance potential: 5.0 V, collision activation dissociation: 10 psi and curtain gas: 20 psi. The compound dependent parameters like declustering potential, collision energy and cell exit potential were optimized at 70 V, 40 eV and 9 V for the analytes and 75 V, 45 eV and 12 V for IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 100 ms. Analyst software version 1.4.2 was used to control all parameters of LC and MS.

### Standard stock, calibration standards and quality control samples

The stock standard solutions of LF and DBL were prepared by dissolving their accurately weighted compounds in methanol to give a final concentration of 200 µg/mL. Further, intermediate solutions (100.0 µg/mL and 50.0 µg/mL) for spiking were prepared in methanol: water (60:40, *v/v*). All the solutions were stored at 2–8 °C and were brought to room temperature before use. The calibration standards (CSs) and quality control (QC) samples were prepared by spiking (5%) blank plasma with standard working solutions. CSs were made at concentration of 5.00, 10.0, 25.0, 50.0, 100.0, 300.0, 600.0, 1200, 2500 and 5000 ng/mL for both the analytes. QC samples were prepared at four different concentration levels, 5.000 (LLOQ, lower limit of quantification quality control), 15.00 (LQC, low quality control), 1000/200.0 (MQC-1/2, medium quality control) and 3500 ng/mL (HQC, high quality control). Stock solution (100.0 µg/mL) of the IS was prepared by dissolving 1.0 mg of artemisinin in 10.0 mL methanol. Its working solution (100 ng/mL) was prepared by appropriate dilution of the stock solution in methanol:water (60:40, *v/v*). All standard stock and working solutions used for

spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

**Protocol for sample preparation**

All frozen subject samples, CSs and QC samples were thawed at room temperature prior to analysis. The samples were adequately vortexed for 10 s. An aliquot of 100 µL plasma sample was mixed with 25 µL of IS. The mixture was vortexed for 2 min, followed by centrifugation at 14000 × g for 5 min at 10 °C. The supernatant was loaded on SPE cartridges which were pre-conditioned with 1.0 mL of methanol followed by 1.0 mL of water. Subsequently, the cartridges were washed with 2.0 mL water and then dried for 2.0 min by applying $1.72 \times 10^5$ Pa pressure at 2.4 L/min flow rate of nitrogen. Elution of analytes and IS from the cartridges was carried out with 100 µL of mobile phase and 5 µL of eluate was used for injection in the LC–MS/MS system.

**Method validation procedures**

The method was validated as per the current regulatory requirements to establish the accuracy and precision of the method [22]. The parameters studied were similar to our previous work [23] and are described in brief.

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of analytes and IS at the start of each batch during method validation. System performance was studied by injecting one extracted blank (without analytes and IS) and one extracted LLOQ sample with IS at the beginning of each analytical batch. Autosampler carryover was evaluated by sequentially injecting extracted blank plasma → ULOQ sample → two extracted blank plasma sample → LLOQ sample → extracted blank plasma at the start and end of each batch.

Selectivity of the method was assessed for potential matrix interferences in ten batches (6 normal lots of $K_3$EDTA, 2 haemolysed, and 2 lipemic) of blank human plasma by extraction and inspection of the resulting chromatograms for interfering peaks. The selectivity of the method toward commonly used medications by human volunteers was also ascertained. This included paracetamol, ranitidine, diclofenac, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 µg/mL) were prepared by dissolving requisite amount in methanol:water (60:40, *v/v*). Further, working solutions were prepared in the mobile phase and 5 µL was injected to check for any possible interference at the retention time of analytes and ISs. The cross talk of multiple reaction monitoring (MRM) for analytes and IS was checked using highest standard on calibration curve and working solution of ISs.

Five calibration lines containing ten non-zero concentrations were used to determine linearity. A quadratic, $1/x^2$, least-squares regression algorithm was used to plot the peak area ratio (analyte/IS) from MRM versus concentration. The linear equations were then used to calculate the predicted concentrations in all samples within the analytical runs. The correlation coefficient for each calibration curve must be ≥0.99 for both the analytes. Reinjection reproducibility for extracted samples was also checked by reinjection of an entire analytical batch after storage at 5 °C.

Intra-day accuracy and precision were evaluated by replicate analysis of plasma samples on the same day. The analytical run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC-1/2 and HQC samples. The inter-day accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days. The precision (% CV) at each concentration level from the nominal concentration should not be greater than 15%. Similarly, the mean accuracy should be within 85–115%, except for the LLOQ where it can be within 80–120 % of the nominal concentration [22].

Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by post column analyte infusion experiment. Briefly, a standard solution containing LF and DBL (at MQC-1 level) was infused post column into the mobile phase at 10 µL/min employing infusion pump. Aliquots of 5 µL of extracted control blank plasma sample were then injected into the column by the autosampler and chromatograms were acquired for both analytes and IS.

Extraction recovery of the analytes and IS from human plasma was evaluated in six replicates by comparing the mean peak area responses of pre-extraction fortified samples to those of post-extraction fortified samples. Matrix effect, expressed as matrix factors (MFs) was assessed by comparing the mean area response of post-spiked samples with samples prepared in mobile phase. IS-normalized MFs (analyte/IS) were calculated to access the variability of the assay due to matrix effects. Relative matrix effect was assessed from the precision (% CV) values of the slopes of the calibration curves prepared from ten different plasma sources, which included haemolysed and lipemic plasma. To prove the absence of matrix interference the % CV should not be greater than 4 %.

Stock solutions of analytes and IS were checked for short term stability at room temperature (25 °C) and long term stability at 5 °C. Stability results in plasma were evaluated by measuring the area ratio response (analyte/IS) of stability samples against freshly prepared comparison standards with identical concentration. Auto sampler (wet extract), bench top (at room temperature), freeze–thaw (at −20 °C and −70 °C) and long term stability (at −20 °C and −70 °C) was performed at LQC and HQC level using six replicates. The stability samples were quantified against freshly prepared quality control samples. Stability data were acceptable if the % change of the replicate determinations did not exceed 15.0 % of the nominal value.

Method ruggedness was verified using two precision and accuracy batches. The first batch was analyzed on two different columns of the same make but different batch number, while the second batch was analyzed by two different analysts who were not part of method validation. The ability to dilute samples which could be above the upper limit of the calibration range was validated by analyzing six replicate samples of 7500 ng/mL and 10000 ng/mL concentration for LF and DBL after two- and ten-fold dilution respectively. The precision and accuracy for dilution reliability was determined by comparing the samples against freshly prepared calibration curve standards.

**Bioequivalence study, statistical analysis and incurred sample reanalysis**

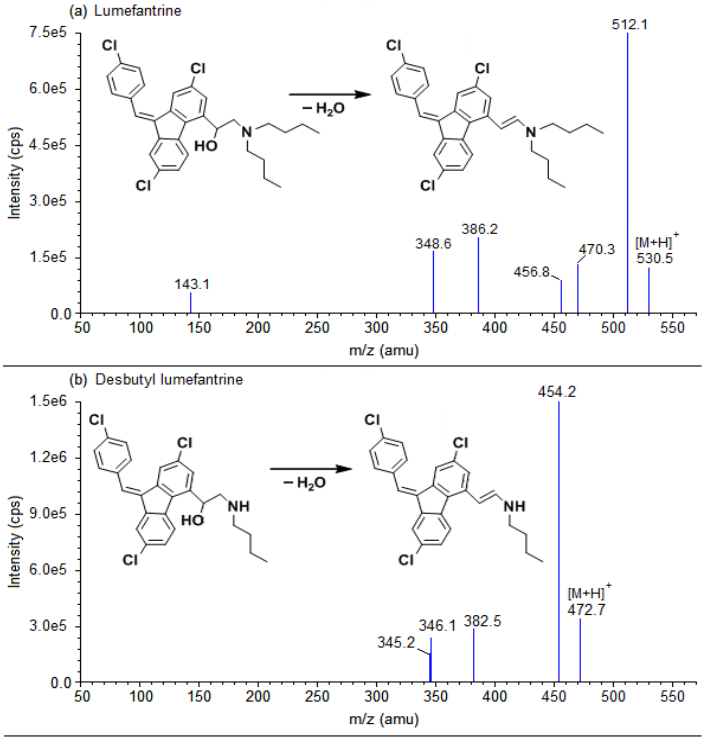
The bioequivalence study was an open label, balanced, randomized, two-treatment, two-period, two-sequence, crossover study for a single dose of test (20/120 mg artemether/lumefantrine tablets from a Generic Indian Company, India) and reference (Coartem® tablets containing 20/120 mg artemether/lumefantrine, marketed by Novartis Pharmaceuticals Corporation, East Hanover, New Jersey, USA) formulation in 12 healthy adult Indian male subjects under fasting. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. The study was conducted strictly in accordance with guidelines laid down by International

Conference on Harmonization and USFDA [24]. The subjects were orally administered a single dose of test and reference formulations with 240 mL water after recommended wash out period of 2 weeks. Blood samples were collected at 0.00 (pre-dose), 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 9.00, 10.0, 12.0, 16.0, 24.0, 48.0, 72.0, 96.0, 144, 192, and 240 h after oral administration of the test and reference formulation in labelled $K_3$EDTA-vacuettes. Plasma was separated by centrifugation and kept frozen at −70 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters were estimated by non-compartmental analysis using WinNonlin® software version 5.3 (Pharsight Corporation, Sunnyvale, CA, USA). To determine whether the test and reference formulations were pharmacokinetically equivalent, $C_{max}$, $AUC_{0-240}$, and $AUC_{0-inf}$ and their ratios (test/reference) using log transformed data were assessed; their means and 90 % CIs were analyzed by using SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA). The drugs were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant ($P \geq 0.05$) and the 90 % confidence intervals (CI) for these parameters fell within 80–125 %.

## RESULT AND DISCUSSION

### Method development

The electrospray ionization provided superior response over atmospheric pressure chemical ionization mode and hence was chosen for the present work. In order to optimize ESI conditions for LF, DBL and artemisinin (IS), quadrupole full scans were carried out in the positive ionization mode. Mass spectrometric conditions like collision energy, cone voltage and capillary voltage were suitably optimized to obtain maximum sensitivity for LF, DBL and IS. The full scan mass spectra for analytes and IS predominantly contained precursor $[M+H]^+$ ions at *m/z* 530.5, 472.7 and 300.2 respectively. The collision induced dissociation mass spectra for LF, DBL and IS revealed most stable and consistent daughter ions at *m/z* 512.1, 454.2, and 219.5 respectively as shown in **Figure 1**.



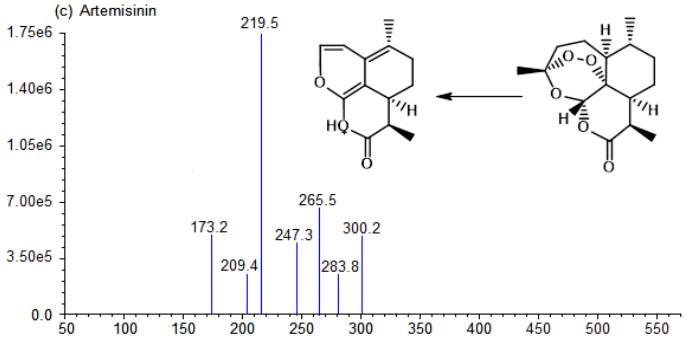




**Figure 1** Product ion mass spectra of (a) lumefantrine (*m/z* 530.5 → 512.1), (b) desbutyl lumefantrine (*m/z* 472.7 → 454.2) and (c) internal standard, artemisinin (*m/z* 300.2 → 219.5) in the scan range 50-570 amu and in the positive ionization mode.

Sample preparation is crucial for reliable quantitation of drugs in biological samples. Several reported methodologies have adopted protein precipitation (PP) [7, 10, 14, 18], liquid-liquid extraction (LLE) [6, 11, 12, 15, 16, 20] or solid phase extraction (SPE) [8, 13] for sample clean-up. Majority of the methods have employed LLE for the simultaneous extraction of LF and DBL from human plasma. Recently, SPE method has been reported for the extraction of LF and DBL from small plasma volume. In the present work, extraction trials were carried out using all three generic techniques but the recovery obtained from SPE was much consistent and quantitative. Lindegårdh et al. [13] used C8 SPE column, however, the recovery for LF and DBL from human plasma was <75 % while Blessborn et al. [8] perform extraction in whole blood. As the goal was to develop a simple and robust method, SPE was carried out on Oasis HLB extraction cartridges (30 mg/1 mL), which required minimal steps for sample cleanup and ensured quantitative and precise recovery at all QC levels for the analytes and IS. For sample processing only 100 µL plasma sample was used and 5 µL was injected into the chromatographic system.

To set the most favorable chromatographic conditions, different buffers like ammonium acetate, ammonium formate in varying combinations with methanol/acetonitrile were tried. Low pH buffer enhanced protonation and helped in eluting the analytes completely without tailing, which assisted in proper quantification of analyte peaks. Number of columns including Hypurity C8 (50 × 4.6 mm, 5 mm), Hypurity Cyano (50 × 4.6 mm, 5 µm), Beta basic Cyano (100 × 2.1 mm, 5 µm), BDS Hypersil C18 (50 × 4.6 mm, 5 µm) and Hypersil Gold C18 (50 × 4.6 mm, 5µm) were evaluated during chromatographic trials. The best chromatography was achieved on Hypersil Gold C18 (50 × 4.6 mm, 5µm) column, which offered well resolved peaks with no peak tailing. The nature of mobile phase and its composition, buffer pH and flow rate was extensively optimized on this analytical column. The best chromatographic conditions were obtained with 5.0 mM ammonium formate (pH 3.0, adjusted with 0.1 % formic acid) and acetonitrile (10:90, *v/v*) as the mobile phase at a flow rate of 1.00 mL/min. These conditions afforded a run time of 2.5 min with retention times of 1.81, 0.90 and 1.15 min for LF, DBL and IS respectively. The MRM chromatograms are depicted in **Figure 2-4**. The capacity factors, which describe the rate at which the analytes migrate through the column, were 2.02 and 0.51 for LF and DBL respectively, while the resolution factor between the analytes was 4.14. The reinjection reproducibility (% CV) of retention times for LF and DBL was ≤ 1.11 for one entire batch on

the same column. Artemisinin, used as IS in the current work gave acceptable results for accuracy and precision at each QC level.

**Assay performance and validation results**

The precision (% CV) of system suitability test was found in the range of 0.19–0.42 % for the retention time and 0.91–1.54 % for the area response for both the analytes and IS. Similarly, the signal to noise ratio for system performance was ≥ 30. There was negligible carry over (≤ 0.18 %) during autosampler carryover experiment. No enhancement in the response was observed in extracted blank plasma (without IS and analytes) following injection of highest calibration standard at the retention time of both the analytes and IS.

All five calibration curves were linear over the concentration range of 5.000 – 5000 ng/mL for LF and DBL with correlation coefficient ($r^2$) ≥ 0.9998. A straight line fit was made through the data points by least square regression analysis to give the mean linear equation $y = (0.00106 \pm 0.000035)\ x - (0.000039 \pm 0.000060)$ and $y = (0.00099 \pm 0.00001)x - (0.000027 \pm 0.000014)$ for LF and DBL respectively, where $y$ is the peak area ratio of the analyte/IS and $x$ the concentration of the analyte.

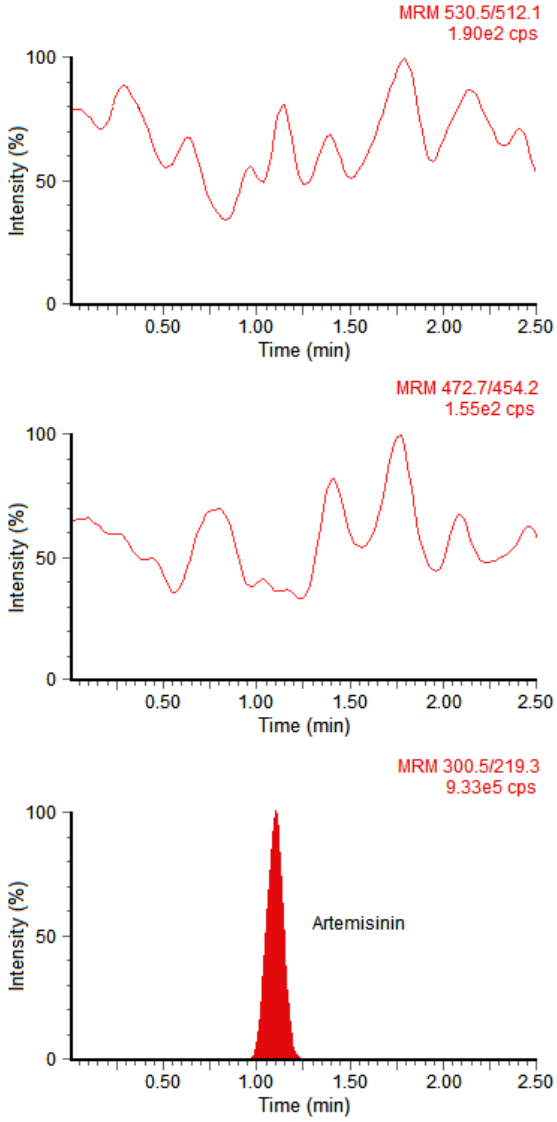



Figure 2 MRM ion-chromatograms of blank plasma for lumefantrine (m/z 530.5 → 512.1), desbutyl lumefantrine (m/z 472.7 → 454.2) and IS (m/z 300.2 → 219.5).

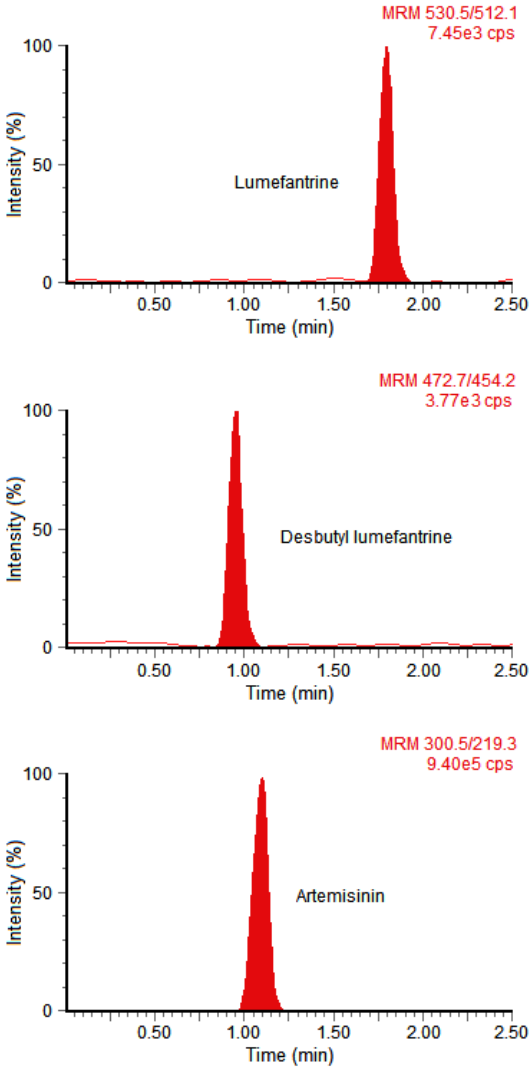
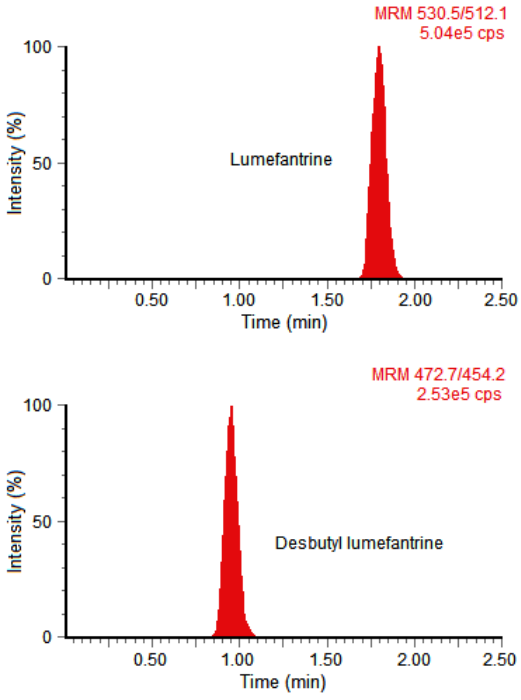



Figure 3 MRM ion-chromatograms of lumefantrine (m/z 530.5 → 512.1) and desbutyl lumefantrine (m/z 472.7 → 454.2) at LLOQ and IS (m/z 300.2 → 219.5).



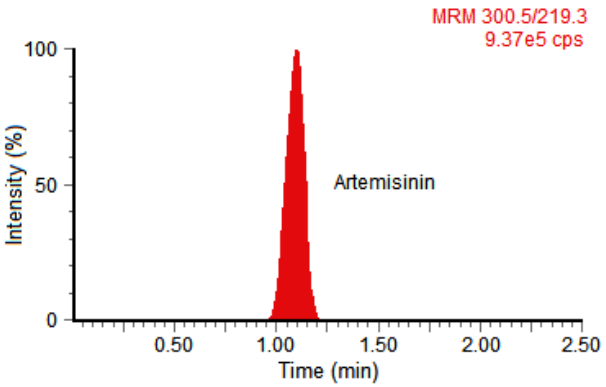



**Figure 4** MRM ion-chromatograms of lumefantrine and desbutyl lumefantrine in subject sample at $C_{max}$ after oral administration of 20/120 artemether/lumefantrine tablet formulation and artemisinin, IS.

The lowest concentration (LLOQ) in the standard curve was 5.000ng/mL for both the analytes in plasma at a signal-to-noise (S/N) ratio ≥ 30. The accuracy and precision (% CV) observed for the calibration curve standards ranged from 96.5 to 103.1 % and 0.29 to 8.82 % for LF and 97.3 to 105.1 % and 1.03 to 3.69 % for DBL, respectively. The analytical method was shown to be selective based on absence of any analytical signals at the retention time of LF, DBL and IS in ten different batches of blank plasma. The intra-batch and inter-batch precision and accuracy results were within the stipulated range of ±15 % of the nominal concentration and < 15 % CV of the mean values as shown in **Table 2**.

Table 2 Intra-day and inter-day precision and accuracy for lumefantrine and desbutyl lumefantrine

| Nominal conc. (ng/mL) | Intra-day (*n* = 6; single batch) | | | Inter-day (*n* = 30; 6 from each batch) | | |
|---|---|---|---|---|---|---|
| | Mean conc. found (ng/mL) | CV (%) | Accuracy (%) | Mean conc. found (ng/mL) | CV (%) | Accuracy (%) |
| *Lumefantrine* | | | | | | |
| HQC (3500) | 3503 | 2.21 | 101.2 | 3573 | 0.96 | 102.1 |
| MQC-1 (1000) | 993.4 | 4.96 | 100.7 | 987.8 | 2.59 | 98.8 |
| MQC-2 (200.0) | 203.2 | 1.77 | 100.2 | 200.9 | 1.66 | 100.4 |
| LQC (15.00) | 14.63 | 7.22 | 99.7 | 14.96 | 3.88 | 99.7 |
| LLOQ QC (5.000) | 4.987 | 6.98 | 98.3 | 5.055 | 3.90 | 101.1 |
| *Desbutyl lumefantrine* | | | | | | |
| HQC (3500) | 3485 | 2.61 | 100.1 | 3437 | 0.57 | 98.2 |
| MQC-1 (1000) | 1030 | 2.51 | 99.3 | 1007 | 4.64 | 100.7 |
| MQC-2 (200.0) | 192.6 | 2.36 | 101.6 | 203.4 | 2.61 | 101.7 |
| LQC (15.00) | 14.86 | 5.01 | 97.5 | 15.05 | 3.41 | 100.3 |
| LLOQ QC (5.000) | 5.084 | 8.12 | 99.7 | 4.952 | 4.42 | 99.0 |

CV: Coefficient of variation; n: Number of replicates; HQC: high quality control; MQC: medium quality control; LQC: low quality control; LLOQ QC: lower limit of quantitation quality control

Table 3 Extraction recovery and matrix factor for lumefantrine and desbutyl lumefantrine

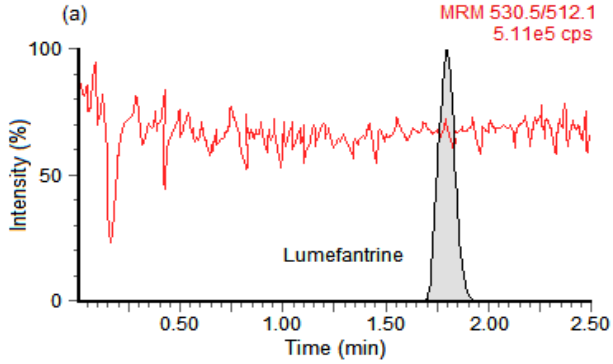
| QC level | Area response (replicate, *n* = 6) | | | Extraction recovery, % (B/A) | | Matrix factor | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | Analyte | IS | Analyte (A/C) | IS | IS-normalized |
| *Lumefantrine* | | | | | | | | |
| LQC | 24122 | 23559 | 23147 | 97.7 | 96.0 | 1.04 | 0.98 | 1.04 |
| MQC-2 | 320587 | 315874 | 317985 | 98.5 | 97.8 | 1.01 | 0.97 | 1.03 |
| MQC-1 | 1513398 | 1489522 | 1548836 | 98.4 | 97.3 | 0.98 | 1.01 | 0.97 |
| HQC | 5633178 | 5591221 | 5640587 | 99.3 | 96.9 | 0.99 | 1.03 | 0.96 |
| *Desbutyl lumefantrine* | | | | | | | | |
| LQC | 20731 | 20477 | 19954 | 98.8 | 96.0 | 1.03 | 0.98 | 1.06 |
| MQC-2 | 279221 | 271021 | 275664 | 97.1 | 97.8 | 1.01 | 0.97 | 1.04 |
| MQC-1 | 1288745 | 1274439 | 1306844 | 98.9 | 97.3 | 0.99 | 1.01 | 0.98 |
| HQC | 4640545 | 4592975 | 4649211 | 98.9 | 96.9 | 0.99 | 1.03 | 0.96 |

A: mean area response of six replicates prepared by spiking in extracted blank plasma; B: mean area response of six replicates prepared by spiking before extraction; C: mean area response of six replicates prepared by spiking in mobile phase (neat samples); IS: internal standard, artemisinin; *n*: number of replicates; LQC: low quality control; MQC: medium quality control; HQC: high quality control.

Matrix effect can be attributed to some undesirable effects that originate from a biological matrix. These components may result in ion suppression/enhancement, decrease/increase in sensitivity of analyte over a period of time, increased baseline, imprecision of data, drift in retention time and distortion or tailing of a chromatographic output. It is suggested that evaluation of matrix factors (MFs) can help to assess the matrix effect. MFs can be determined from the peak area response for the analyte and IS separately, while the ratio of the two factors yields IS-normalized MF. Further, matrix effect needs to be checked in lipemic and haemolysed plasma samples in addition to normal $K_3$EDTA plasma. The extraction recovery and matrix factors for LF and DBL are presented in **Table 3**. The mean extraction recovery for LF, DBL and IS were 98.5, 98.4 and 90.7 % respectively. Further, the relative matrix effect expressed as precision (% CV) in the measurement of the slopes of the calibration curves was < 3.1 % in ten different plasma sources.

Further, qualitative assessment of matrix effect through post-column infusion experiment showed no ion suppression or enhancement at the retention time of LF, DBL and IS in the chromatograms (**Figure 5).**

The stability of analyte and IS in human plasma and stock solutions was examined under different storage conditions. Stock solutions for short term stability of LF, DBL and IS were stable at room temperature up to 24 h and between 2-8 °C for a minimum period of 30 days. LF and DBL in control human plasma (bench top) at room temperature was stable for at least 20 h at 25 °C and for minimum of six freeze and thaw cycles. Autosampler (processed sample) stability of the spiked quality control samples was determined up to 36 h. Long term stability of the spiked quality control samples remained unchanged up to 198 days. The % change values for different stability experiments at LQC and HQC levels in plasma are shown in **Table 4**.



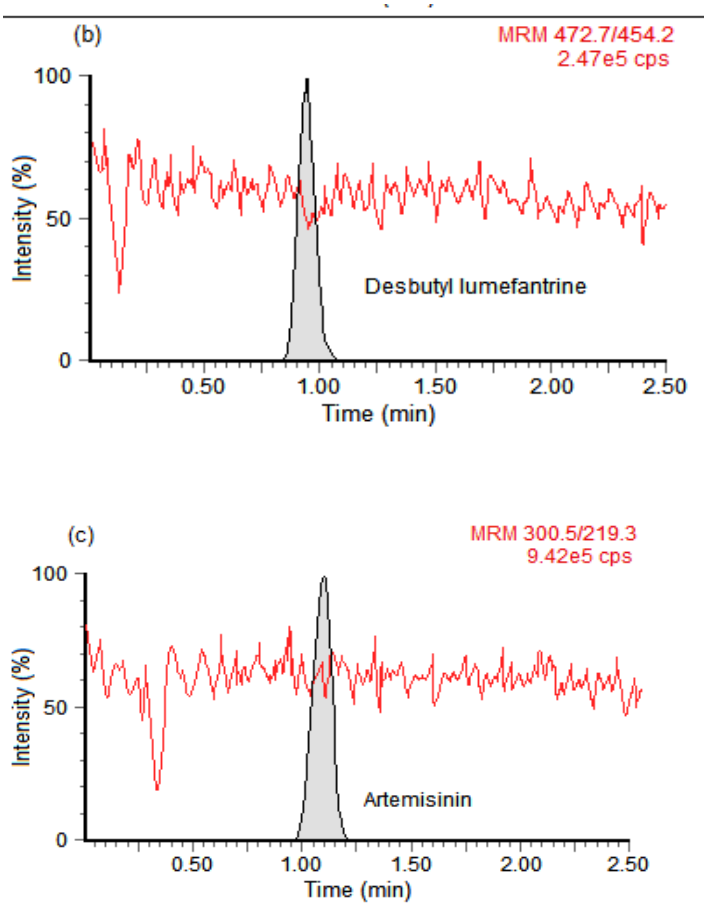




**Figure 5** Post column analyte infusion MRM LC-MS/MS chromatograms for (a) lumefantrine, (b) desbutyl lumefantrine and (c) artemisinin.

The ruggedness of the method was evaluated by re-injection of analyzed samples on two Hypersil Gold C18 (50 × 4.6 mm, 5.0 µm) columns, each from a different batch and also by two analysts. The precision (% CV) and accuracy values for different columns and analysts ranged from 0.5 to 2.5 % and from 95.1 to 103.1 % respectively for LF and DBL across five QC levels. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision values for dilution reliability for 1/5 and 1/10th dilution were within 0.7 to 1.8%, while the accuracy results were between 96.3 % and 97.1 % respectively.

Table 4 Stability results of lumefantrine and desbutyl lumefantrinein plasma under various conditions (n = 6)

| **Storage conditions** | **Nominal conc. (ng/mL)** | **Mean stability sample (ng/mL ± SD)** | **Change (%)** | **Mean stability sample (ng/mL ± SD)** | **Change (%)** |
|---|---|---|---|---|---|
| | | *Lumefantrine* | | *Desbutyl lumefantrine* | |
| Bench top stability at 25 °C, 20 h | 3500 | 3518 ± 75.4 | 0.51 | 3476 ± 61.8 | -0.66 |
| | 15.00 | 15.15 ± 0.45 | 0.97 | 14.79 ± 0.69 | -1.38 |
| Freeze & thaw stability at -20 °C | 3500 | 3410 ± 52.3 | -2.56 | 3469 ± 40.4 | -0.88 |
| | 15.00 | 14.61 ± 0.32 | -2.59 | 15.93 ± 0.60 | 6.21 |
| Freeze & thaw stability at -70 °C | 3500 | 3466 ± 50.1 | -0.97 | 3457 ± 68.5 | -1.24 |
| | 15.00 | 14.28 ± 0.22 | -4.82 | 14.87 ± 0.79 | -0.90 |

| | | | | | |
|---|---|---|---|---|---|
| Autosampler stability at 4°C, 36 h | 3500 | 3478 ± 61.3 | -0.63 | 3444 ± 66.40 | -1.60 |
| | 15.00 | 14.77 ± 0.67 | -1.56 | 14.48 ± 0.96 | -3.44 |
| Long term stability at -20 °C, 198 days | 3500 | 3145 ± 66.9 | -2.44 | 3477 ± 91.3 | -0.65 |
| | 15.00 | 16.31 ± 0.90 | 8.73 | 15.34 ± 0.35 | 2.26 |
| Long term stability at -70 °C, 198 days | 3500 | 3435 ± 32.5 | -1.85 | 3519 ± 36.1 | 0.54 |
| | 15.00 | 14.57 ± 0.50 | -2.88 | 14.35 ± 0.37 | -4.36 |

SD: standard deviation; *n*: number of replicates; HQC: high quality control; LQC: low quality control

$$\%\text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$$

**Application of the method in healthy human subjects**

The validated method was successfully applied for the assay of LF and DBL in 12 healthy Indian male subjects. **Figure 6** shows the plasma concentration vs. time profile for LF and DBL under fasting condition.

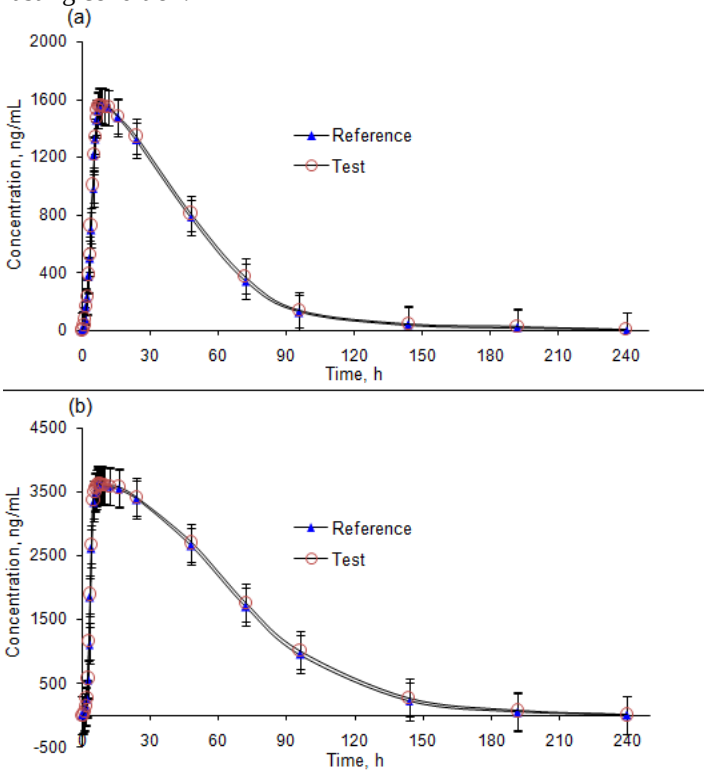



**Figure 6** Mean plasma concentration-time profile of lumefantrine and desbutyl lumefantrine after oral administration of test (20/120 mg tablets from a Generic Indian Company, India) with a reference (Coartem® tablets containing 20/120 mg artemether/lumefantrine tablets from Novartis Pharmaceuticals Corporation, East Hanover, New Jersey, USA) formulation to 12 healthy subjects.

Approximately 672 samples including the calibration and QC samples with volunteer samples were run and analyzed during a period of 5 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The important pharmacokinetic parameters namely, maximum plasma concentration ($C_{max}$), area under the plasma concentration-time curve from 0 to 240 h ($AUC_{0\text{-}240}$), area under the plasma concentration-time curve from zero hour to infinity ($AUC_{0\text{-}inf}$), time point of maximum plasma concentration ($T_{max}$), half life of drug elimination during the terminal phase ($t_{1/2}$) and elimination rate constant ($K_{el}$) were calculated for the test and reference formulations and are presented in **Table 5**.

**Table 5** Mean pharmacokinetic parameters after oral administration of 20/120 mg artemether/ lumefantrine tablet formulation in 12 healthy Indian subjects under fasting.

| Parameter | Test (Mean ±SD) | Reference (Mean ±SD) | Ratio (test/reference),% |
|---|---|---|---|
| *Lumefantrine* | | | |
| $C_{max}$ (ng/mL) | 1561.25 ± 532.49 | 1560.66 ± 614.18 | 100.03 |
| $AUC_{0\text{-}240\ h}$ (h.ng/mL) | 83214.9 ± 985.5 | 79751.0 ± 1021.2 | 104.34 |
| $AUC_{0\text{-}inf}$ (h.ng/mL) | 89815.5 ± 1009.3 | 85644.8 ± 1011.8 | 104.85 |
| $T_{max}$ (h) | 7.25 ± 1.45 | 7.12 ± 1.22 | --- |
| $t_{1/2}$ (h) | 58.67 ± 15.39 | 55.71 ± 18.11 | --- |
| $K_{el}$ (1/h) | 0.0118 ± 0.0090 | 0.0124 ± 0.0061 | --- |
| *Desbutyl lumefantrine* | | | |
| $C_{max}$ (ng/mL) | 3620.21 ± 711.27 | 3611.13 ± 695.66 | 100.25 |
| $AUC_{0\text{-}240\ h}$ (h.ng/mL) | 275519.9 ± 1055.4 | 265412.7 ± 1021.1 | 103.80 |

| | | | |
|---|---|---|---|
| $AUC_{0\text{-}inf}$ (h.ng/mL) | 339125.5 ± 1335.1 | 327452.2 ± 1524.9 | 103.56 |
| $T_{max}$ (h) | 7.05 ± 1.07 | 6.90 ± 1.13 | --- |
| $t_{1/2}$ (h) | 75.85 ± 8.36 | 72.33 ± 9.39 | --- |
| $K_{el}$ (1/h) | 0.0092 ± 0.0001 | 0.0096 ± 0.0002 | --- |

SD: standard deviation; CV: coefficient of variation

## CONCLUSIONS

The bioanalytical methodology developed for the simultaneous determination of LF and DBL is highly specific and rugged for therapeutic drug monitoring. It can be readily applied for the analysis of routine samples with desired sensitivity, precision, accuracy and high throughput. The method involved a simple, quick, clean and specific sample preparation by solid phase extraction. A short analysis time of 2.5 min under isocratic conditions ensures higher throughput for subject sample analysis compared to reported methods in the literature. The method was shown to be selective and free from matrix interference as evident from the results of post-column infusion, IS-normalized matrix factors and relative matrix effect in different plasma sources. Moreover, the established LLOQ is adequate enough to conduct a pharmacokinetic study/bioequivalence study with 20/120mg formulation of artemether/lumefantrine in healthy human volunteers.

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**Citation:** Pranav S. Shrivastav *et al*. (2015). Bioanalytical Method Development and Validation for the Estimation of Lumefantrine and Desbutyl Lumefantrine in Human Plasma by LC-MS/MS J. of Advancement in Medical and Life Sciences. V3I4. DOI: 10.15297/JALS.V3I4.02