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Bio-Analytical Method Development and Validation for Estimation of Lume fantrine in Human Plasma by Using Lc-Ms/Ms

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Abstract

Lumefantrine and Glimepiride (IS) were extracted from human plasma by Precipitation followed by Solid phase extraction using Orochem (30 mg/1 CC) solid phase extraction cartridge. The chromatographic separation was performed on Hypurity C18 (50 cm×4.6 mm), 5 µ column. The mobile phase consisted of Acetonitrile: 2 mM Ammonium Acetate (pH: 3.5) (90:10, % v/v) was delivered at rate of 0.600 mL/min with Splitter. Detection and quantitation were performed by a triple quadrupole equipped with electro spray ionization and multiple reaction monitoring in positive ionization mode (API 3000). The most intense [M-H]- transition for Lume fantrine at m/z 528.0→510.0 and for IS at m/z 491.2→352.0 were used for quantification. The developed method was successfully applied for bioequivalence study of Lume fantrine. The method was found to linear over the range of 100-20000 ng/mL (r≥0.992). The lower limit of quantitation (LLOQ) was 100 ng/mL. The extraction recovery was above 75% for analyte and above 90% for IS. The intra and inter-day accuracy was found to 92.27% - 104.00%. The intra and inter-day precision expressed as % CV were 1.76% - 6.47%, respectively. The stability testing was also investigated and it was found that both drug and IS were quite stable. A simple, rapid, sensitive, accurate and precise LC-MS/MS method has been developed for the quantification of Lumefantrine from human plasma using Protein precipitation followed by SPE method. The method exhibited good linear response over the selected concentration range 100-20000 ng/mL. Selectivity and sensitivity were sufficient for detecting and quantifying Lumefantrine in human plasma. These features coupled with a short run time at 3.50 min compared to reported methods, facilitated a high analysis throughput, with the ability to quantify a larger number of clinical samples in a shorter time frame.

Keywords: Malaria; Bioequivalence; Plasma; Centrifugation; Ionization; Optimization; Matrix

Introduction

Lumefantrine (IUPAC name: 2-(dibutylamino)-1-[(9Z)-2, 7-dichloro-9-(4-chlorobenzylidane)-9H-fluoren-4-yl] ethan-1-ol) is an antimalarial agent used to treat acute uncomplicated malaria. It is administered in combination with artemether for improved efficacy. This combination therapy exerts its effects against the erythrocytic stages of *Plasmodium* and may be used to treat infections caused by P. falciparum and unidentified plasmodium species, including infections acquired in chloroquine-resistant areas [1]. Few bioanalytical methods are reported to determine lumefantrine in tablet using HPLC-UV [2], in plasma HPLC [3], HPLC [4,5] and LC-MS/MS [6] detection. All reported methods have long run time. Hence, it felt necessary to develop and validate a rapid and selective method that can be successfully applied to a bioequivalence study. In the present paper we would like to present a simple and high-throughput protein precipitation method for quantification of lumefantrine using Glimipiride as an internal standard with LC-MS/MS detection. The application of this validated method in analyzing samples from a bioequivalence study involving lumefantrine is also presented.

Experimental Conditions

Chemicals and reagents

The reference standard of lumefantrine was provided by accutest pharmaceuticals Ltd. (Mumbai, India). The reference standard Glimepiride was obtained from accutest pharmaceuticals Ltd. Purity of both the standards was higher than 99%. The lumefantrine tablets, containing 120 mg lumefantrine per tablet, were obtained from Novartis Pharmaceuticals Corp., Suffern, New York. High-purity water was prepared in-house using a Milli-Q A10-gradient water purification system (Millipore, Bangalore, India). LC-grade methanol and acetonitrile were purchased from J.T. Baker Inc, and sigma, germany. (Phillipsburg, NJ, USA). HPLC-grade acetic acid was procured from Merck (Mumbai, India). Drug-free (blank) human plasma containing heparin was obtained by enrolling healthy volunteers and taking their consent before bleeding. The plasma thus obtained was stored at −20°C prior to use.

Calibration curve and quality control samples

Two separate stock solutions of lumefantrine were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise as well as the subject sample analysis. The stock solutions of lumefantrine and Glimepiride were prepared in Acetonitrile: 2 mM Ammonium Acetate (pH:3.5) (90:10, % v/v) at free base concentration of 2500 µg/mL. Primary dilutions and working standard solutions were prepared from stock solutions using Methanol: Water (80:20 v/v) solvent mixture. These working standard solutions were used to prepare the calibration curve and quality control samples. Blank human plasma was screened prior to spiking to ensure that it was free of endogenous interference at retention times of lumefantrine

and internal standard Glimepiride. An eight-point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of lumefantrine. Calibration samples were made at concentrations of 100, 200, 1000, 2000, 4000, 10000, 14000, and 20000 ng/mL, and quality control samples were made at concentrations of 280, 9000 and 15000 ng/mL for lumefantrine.

Liquid chromatography and mass spectrometric conditions

The chromatographic separation was performed on Hypurity C18 (50 cm×4.6 mm), 5 µ column. The mobile phase consisted of Acetonitrile: 2mM Ammonium Acetate (pH:3.5) (90:10, %v/v) was delivered at rate of 0.600 mL/min with Splitter. Detection and quantitation were performed by a triple quadrupole equipped with electrospray ionization and multiple reaction monitoring in positive ionization mode (API 3000). The tuning was performed with ion-spray voltage 2500 eV, heater gas flow 8000 lit/hr and temperature was 400°C. The most intense $[M-H]^-$ transition for Lumefantrine at m/z 528.0→510.0 and for IS at m/z 491.2→352.0 were used for quantification. The developed method was successfully applied for bioequivalence study of Lumefantrine. The method was found to linear over the range of 100-20000 ng/mL ($r \geq 0.992$). The lower limit of quantitation (LLOQ) was 100 ng/mL. Selectivity and sensitivity were sufficient for detecting and quantifying Lumefantrine in human plasma. These features coupled with a short run time at 3.50 min compared to reported methods, facilitated a high analysis throughput, with the ability to quantify a larger number of clinical samples in a shorter time frame.

The data acquisition was ascertained by Analyst 1.4.1 software. For quantification, the peak area ratios of the target ions of the analyte to those of the internal standard were compared with weighted $(1/x^2)$ least squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations (Figures 1 and 2).

Plasma sample preparation

To 100 µl of analyte free plasma in micro tube 1 ml precipitating agent was added and vertex to mix. Then after 25 µl Internal Standard was added and vertex to mix. The samples were then centrifuged for 5 minutes at 14000 rpm and 5 ml of supernatant layer was injected into the LC-MS/MS system through the auto sampler.

Validation

A thorough and complete method validation of lumefantrine in human plasma was carried out following US FDA guidelines. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, dilution integrity, partial volume, reinjection reproducibility, and stability. Selectivity was performed by analyzing the human blank plasma samples from six different sources (or donors) with an additional haemolysed group and lipemic group to test for interference at the retention times of analytes. The assessment of matrix effect (coeluting, undetected endogenous matrix compounds that may influence the analyte ionization) constitutes an important and integral part of validation for quantitative LC-MS/MS method for supporting pharmacokinetics studies. It was performed by processing six different lots of plasma samples in quadruplet. LQC and HQC working solutions were spiked following extraction in duplicate for each lot. The % CV at each level was calculated by taking the mean value obtained by injecting the post extracted samples prepared in duplicate from each plasma lot, which should be less than ten. The intra-run (within a day, and inter-run (between days, accuracy was determined by replicate analysis of quality control samples (at LLOQ (lower limit of quantification), LQC (low quality control), MQC (medium quality control), HQC (high quality control), and ULOQ (upper limit of quantification) levels. The % CV should be less than 15% and accuracy (% RE) should be within 15% except LLOQ where it should be within 20%. Accuracy is defined as the percent relative error (% RE) and was calculated using the formula % RE=$((E-T)/T)$ 100, where E is the experimentally determined concentration and T is the theoretical concentration. Assay precision was calculated by using the formula % CV=(SD/M) (100), where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M. The
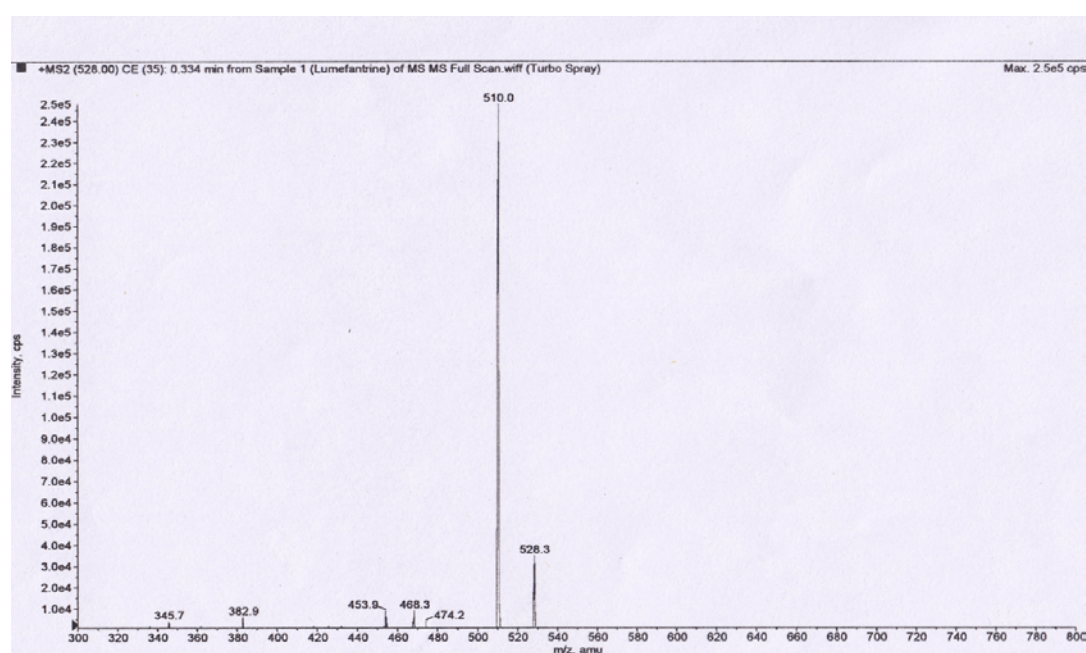

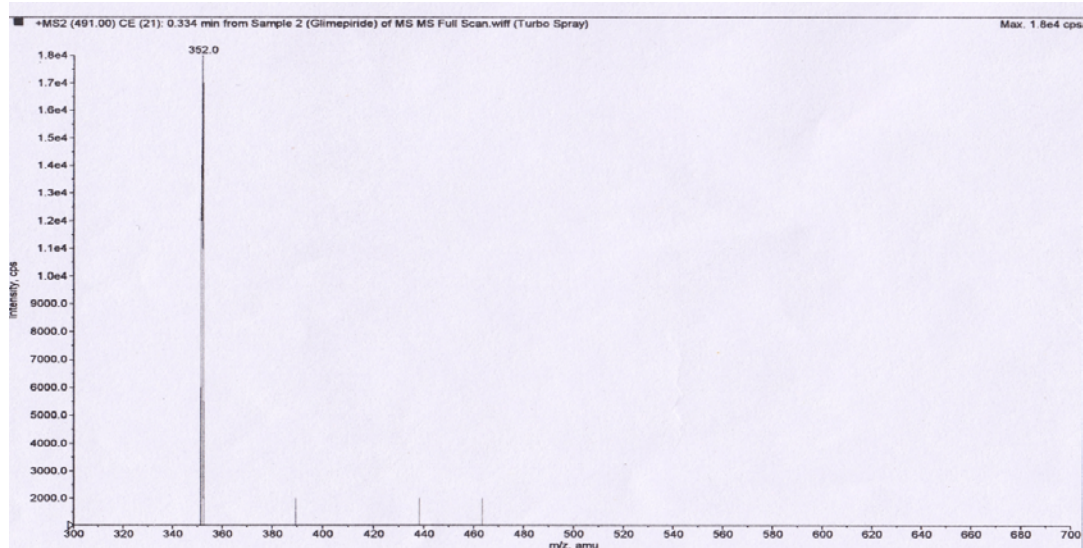**Figure 1:** Product ion mass spectrum of lumefantrine.

Figure 2: Product ion mass spectrum of glimepride.

% change was calculated by using the formula % change=(S/F−1) 100, where S is the mean concentration of stability samples and F is the mean concentration of freshly prepared samples. The extraction efficiencies of lumefantrine and Glimipiride were determined by analysis of six replicates at each quality control concentration level for lumefantrine and at one concentration for the internal standard Glimipiride. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of unextracted standards (spiked into extracted matrix of same lot). The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. Dilution integrity experiment was carried out at 1.7 times the ULOQ concentration. Six replicates each of 1/2 and 1/4 concentrations were prepared and their concentrations were calculated by applying the dilution factor 2 and 4 against the freshly prepared calibration curve. In real subject samples with insufficient plasma volume, the partial volume experiment was performed on medium quality control (MQC) concentration level to validate the method. Six replicates each of half and quarter volume of the total volume of plasma required for processing were prepared and their concentrations were calculated by applying the concentration factor 2 and 4 against the freshly prepared calibration curve. LQC and HQC samples were injected to check re-injection reproducibility, after which the system was turned off and then restarted after two hours. The same samples were then reinjected, and original values were compared with re-injected values with respect to % change, which should be less than 10%. As a part of the method validation, stability was evaluated in stock solutions and in plasma under different conditions, maintaining the same conditions that occurred during study samples handling and analysis. Stock solution stability was performed by comparing area response of the analyte and the internal standard in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the % change is less than 15% as per US FDA guidelines.

The stability of the spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 4 to 24 hrs. The stability of the spiked human plasma samples stored at −70°C in coolant (coolant stability) was evaluated for 24 h. The auto sampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 hrs), with the samples that were re-injected after storing in the auto sampler at 5°C for 52 hrs. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the refrigerator at 2-8°C for 19 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at −70°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long-term stability evaluation, freshly prepared calibration curve and quality control samples were injected along with the stability samples. The concentrations obtained after 16, 39, 87, and 221 days intervals were compared with initial concentrations.

Application of Method

The validated method has been successfully used to analyze lumefantrine concentrations in sixty human volunteers under fasting conditions after administration of a single tablet containing 120 mg lumefantrine as an oral dose. The study design was a randomized, two-period, two-sequence, two-treatment single-dose, open-label, bioequivalence study using COARTEM manufactured by Novartis Pharmaceuticals Corp, as the reference formulation. The study was conducted according to current GCP guidelines and after signed consent of the volunteers. Before conducting the study, it was also approved by an authorized ethics committee. There were a total of 25 blood collection time-points including the predose sample, per period. The blood samples were collected in separate vacutainers containing heparin as anticoagulant. The plasma from these samples was separated by centrifugation at 3500 rpm within the range of 2-8°C. The plasma samples thus obtained were stored at −70°C till analysis. Following analysis the pharmacokinetic parameters were computed using WinNonlin software version 5.2 and 90% confidence interval was computed using SAS software version 9.2.

Results and Discussion

Method development

During method development different options were evaluated to optimize detection parameters, chromatography, and sample extraction.

Mass spectra

Electro spray ionization (ESI) provided maximum response over atmospheric pressure chemical ionization (APCI) mode and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at both polarities at a flow rate of 8000 L/hrs. For the optimization of tuning parameter of Lumefantrine and Glimepiride (IS), I was tried to changing the different values of Dp, FP, EP, CE and CXP, but parent peak of Lumefantrine and Glimepiride was not found 528 amu and 491.2 amu respectively, product peak of Lumefantrine and Glimepiride was not found 510 amu and 352 amu respectively. Finally DP=50, FP=330, EP=13, CE=35 and CXP=14 for Lumefantrine and DP=40, FP=280, EP=12.50, CE=21 and CXP=23 for Glimepiride was optimize which gives the Lumefantrine Parent peak at 528 amu and Lumefantrine product peak at 510 amu and Glimipiride Parent peak at 491.2 amu and Glimipiride Product peak at 352 amu.

Chromatography

In Aqueous sample for the optimization of mobile phase, I was tried to different mobile phase with changing its composition. But satisfaction and good result was not found, finally ACN: 2 mM Ammonium Acetate pH:3.5 (90:10%v/v) was optimize which gives good peak shape, reproducibility, good separation and no impurity found. In Plasma for the optimization of mobile phase, I was tried to different mobile phase with changing its composition. But satisfaction and good result was not found, finally 2 mM Amm. Acetate in Water pH = 3.50 ± 0.05, Buffer: CAN (10:90%v/v) was optimize which gives constant Peak area, recovery was good and separation was good, No impurity was found.

Extraction

Several organic solvents were employed to extract analytes from the plasma sample. All the tested solvents (ethyl acetate, chloroform, hexane, dichloromethane, and methyl tertiary butyl ether) in liquid-liquid extraction yield less recovery. Protein precipitation using acetonitrile and mobile phase was also tried. As compared to the acetonitrile, mobile phase yields high recovery. It was difficult to find a compound which could ideally mirror the analytes to serve as a good IS. Several compounds were investigated to find a suitable IS, and finally Glimipiride belonging to a similar class of compounds was found to be most appropriate for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity, or ion suppression. The results of method validation using Glimipiride as the IS were acceptable in this study based on FDA guidelines. High recovery and selectivity were observed in the protein precipitation method. These optimized detection parameters, chromatographic conditions, and extraction procedure resulted in reduced analysis time with accurate and precise detection of lumefantrine in human plasma.

Method Validation

Selectivity and sensitivity

Representative chromatograms obtained from blank plasma, plasma spiked with lower limit of quantification, and real subject sample for lumefantrine and Glimipiride are shown in Figure 3. The mean % interference observed at the retention time of analytes between eight different lots of human plasma including haemolysed and lipemic plasma containing heparin as an anticoagulant was calculated and the value was found to be 0.00% and 0.00% for lumefantrine and Glimipiride, respectively, which was within acceptance criteria. Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least interference at the retention time of lumefantrine were prepared and analyzed. The % CV of the area ratios of these six replicates of samples was 2.68% for lumefantrine confirming that interference does not affect the quantification at the LLOQ level. Utilization of selected product ions for each compound enhanced mass spectrometric selectivity.

The LLOQ for lumefantrine was 100 ng/mL. The intra-run precision and intra-run accuracy (% CV) of the LLOQ plasma samples containing lumefantrine were 1.76% to 4.49%, respectively. All the values obtained below 100 ng/mL for lumefantrine were excluded from statistical analysis as they were below the LLOQ values validated for lumefantrine.

Matrix effect

The assessment of matrix effect constitutes an important and integral part of validation for quantitative LC-MS/MS for supporting pharmacokinetic studies. It was performed by processing six different lots of plasma samples in quadruplet (LQC and HQC working solutions were spiked after extraction in duplicate for each lot. The results found were well within the acceptable limits as the % CV of the area ratios of posts piked recovery samples at LQC and HQC was 4.49% and 2.96, respectively, which was within 10% for lumefantrine. Hence minor suppression or enhancement of analytes signal due to endogenous matrix interferences did not affect the quantification of lumefantrine.

Linearity, precision and accuracy, and recovery

The peak area ratios of calibration standards were proportional to the concentration of lumefantrine in each assay over the nominal concentration range of 100–20000 ng/mL. The calibration curves appeared linear and were well described by least-squares linear regression lines. As compared with the 1/x weighing factor, a weighing factor of $1/x^2$ properly achieved the homogeneity of variance and was chosen to achieve homogeneity of variance. The regression squares were greater than 0.9992 for lumefantrine. The deviation of the back calculated values from the nominal standard concentrations were less than 15%. This validated linearity range justifies the concentration observed during real sample analysis. The inter-run precision and accuracy were determined by pooling all individual assay results of replicate () quality control over three separate batch runs analyzed on three different days. The inter-run precision (% CV) and inter-run accuracy (% RE) at LLOQ level were 2.96% to 6.47% respectively for lumefantrine. The intra-run precision and accuracy were determined by pooling all individual assay results of replicate () quality control of two separate batch runs analyzed on the same day. The intra-run precision (% CV) and intra-run accuracy (% RE) at LLOQ level were 1.76% to 4.49%, respectively, for lumefantrine. The intra-run and inter-run precision and accuracy data at all quality control level are presented in Table 1. Both the (% CV) precision and accuracy (% RE) at all quality control levels were within 15%, which indicates that the method is precise and accurate.

Six post extracted replicates (samples spiked in extracted matrix

Run	Concentration added (ng/mL)	Mean concentration found (ng/mL)	% CV	% RE
Intra-	102.000	100.777	1.76	-4.76
	280.000	291.198	4.49	-5.19
	9000.000	8668.107	2.26	-1.16
	15000.000	13839.853	2.96	-2.56
Inter-	102.000	105.717	6.47	-4.47
	280.000	290.735	4.67	-5.67
	9000.000	8802.353	3.69	-1.59
	15000.000	13958.249	2.96	-3.76

CV: coefficient of variation; RE: relative error

Table 1: Intrarun and interrun precision and accuracy (n=6) of lumefantrine in human plasma.

Analytes	Level	A	B	% Recovery	% CV
Lumefantrine	LQC	1979	1442	72.88	5.03
	MQC	55693	40731	73.16	3.65
	HQC	89231	71804	80.48	5.32
glimipiride	LQC	59763	58081	97.25	5.17
	MQC	63211	54919	86.89	2.92
	HQC	63268	61438	97.18	6.98

A: mean area of unextracted sample (n=6); B: mean area of extracted sample(n=6); mean recovery was found to be 75.50% for lumefantrine and 93.77 for Glimipiride; CV: coefficient of variation.

Table 2: Recovery for lumefantrine and Glimipiride (n=6).

of same lot) at low, medium, middle, and high quality control concentration levels for lumefantrine were prepared for recovery determination, and the areas obtained were compared versus the areas obtained for extracted samples (Table 2) of the same concentration levels from a precision and accuracy batch run on the same day.

Dilution integrity and partial volume

Dilution integrity and partial volume exercise was performed using six replicates of respective samples. The mean back calculated concentrations for 1/2 and 1/4 dilution samples were within 85-115% of their nominal. The % CV for 1/2 and 1/4 dilution samples were 3.56% and 3.33% respectively. The mean back calculated concentrations for half and quarter partial volume samples were within 85-115% of their nominal. The % CV for half and quarter partial volume samples was 2.38% and 1.89%, respectively.

Reinjection reproducibility and stabilities

Reinjection reproducibility exercise was performed to check whether the instrument performance remains unchanged after hardware deactivation due to any instrument failure during real subject sample analysis. % Change was less than 9.25% for LQC and HQC level concentration; hence batch can be reinjected in case of instrument failure during real subject sample analysis. Also samples prepared were reinjected after 29 hours which shows % change less than 11.61% for LQC and HQC level concentration; hence the batch can be reinjected after 29 hours in case of instrument failure during real subject sample analysis. Stock solution stability was performed to check stability of lumefantrine and Glimipiride in stock solutions prepared in Acetonitrile: 2 mM Ammonium Acetate (pH:3.5) (90:10, % v/v) and stored at 2-8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 16 days. The % change for lumefantrine and Glimipiride was 0.58% and 0.65%, respectively, indicating that stock solutions were stable at least for 16 days. Bench-top, coolant and auto sampler stability for lumefantrine was investigated at LQC and HQC levels. The results

revealed that lumefantrine was stable in plasma for at least 17 h at room temperature, 26 h in a coolant at -70°C, and 34 h in an auto sampler at 10°C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with lumefantrine at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that lumefantrine was stable in matrix up to 221 days at a storage temperature of −70°C. The results obtained from all these stability studies are tabulated in Table 3.

Application

The validated method has been successfully used to quantify lumefantrine concentrations in sixty human volunteers, under fasting conditions after administration of a single tablet containing 120 mg lumefantrine as an oral dose. The study was carried out after approval from an independent ethics committee and after obtaining signed approval from the volunteers.

The pharmacokinetic parameters evaluated were C_{max} (maximum observed drug concentration during the study), AUC_{0-72} (area under the plasma concentration-time curve measured 72 hours, using the trapezoidal rule), T_{max} (time to observe maximum drug concentration), Kel (apparent first-order terminal rate constant calculated from a semi log plot of the plasma concentration versus time curve, using the method of least squares regression), and $T_{1/2}$ (terminal half-life as determined by quotient 0.693/Kel). The mean C_{max} that was observed for lumefantrine in case of both test and reference formulations was 4078.66 and 4179.93 ng/mL, respectively. The corresponding mean that was observed for lumefantrine in case of both test and reference formulations was 1.70 and 1.47 h. The mean AUC_{0-72} that was observed for lumefantrine in case of both test and reference formulations was 26265.54 and 26451.19 ng h/mL, respectively The 90% confidence intervals of the ratios of means C_{max}, AUC_{0-72} all fell within the acceptance range of 80%-125%, demonstrating the bioequivalence of the two formulations of lumefantrine.

Conclusion

The method for estimation of Lumefantrine from human plasma by LC-MS/MS was validated for performance characteristics related to accuracy, precision, linearity, selectivity, stability of the drug in biological matrix at ambient and at freezing conditions, stability of drug in extracted media. The method is linear over the concentration studied i.e. 100.00 ng/ml to 15000.00 ng/ml of Lumefantrine .The method is selective in presence of plasma interferences.

Stability	Level	A	% CV	B	% CV	% Change
Autosampler (34 h, 10°C)	LQC	302.085	5.50	295.844	4.23	-2.42
	HQC	14054.185	3.31	15432.059	2.27	9.39
Bench top (17 h at room temp.)	LQC	306.157	3.09	298.257	2.63	-2.58
	HQC	14505.513	5.32	13932.784	7.01	-4.11
Coolant (26 h, −70°C)	LQC	307.762	2.17	308.037	2.15	0.65
	HQC	13862.074	5.34	15405.576	1.49	10.61
Reinjection (29 h, 2–8°C)	LQC	303.085	5.90	294.844	4.43	-2.72
	HQC	14034.185	3.81	15492.059	2.77	10.39
3rd freeze-thaw cycle (−70°C)	LQC	303.762	2.07	306.037	2.10	0.75
	HQC	13812.074	5.14	15415.576	1.59	11.61
Long term (221 days, −70°C)	LQC	303.085	5.90	305.112	3.40	0.67
	HQC	14034.185	3.81	15234.846	1.51	8.56

A: mean value of comparison samples (original concentrations before storage) concentrations (ng/mL); B: mean value of stability samples (measured concentration after storage) concentrations (ng/mL); CV: coefficient of variation; h: hours, temp: temperature.

Table 3: Stability results for lumefantrine (n=6).
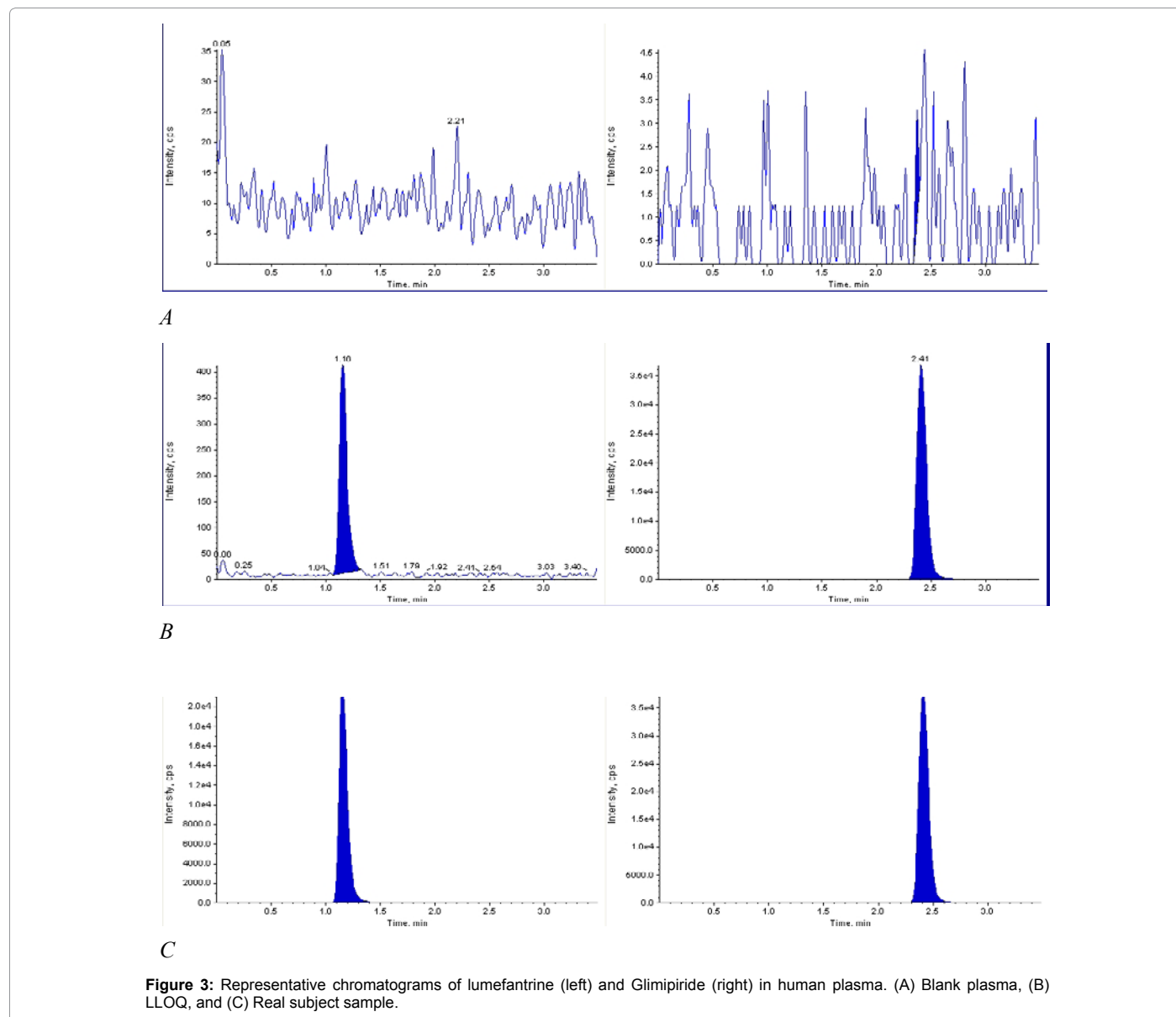
Figure 3: Representative chromatograms of lumefantrine (left) and Glimipiride (right) in human plasma. (A) Blank plasma, (B) LLOQ, and (C) Real subject sample.

Interference is insignificant. Recovery is consistent at higher middle and at lower level. % CV for recovery is well within acceptance criteria for all three QC levels studied. The results are precise for all three sets of QC and for LLOQ. Accuracy also holds well with all replicates of all QC levels and LLOQ lying within acceptance criteria for back-calculated concentration. Precision and accuracy also holds good on intra batch basis. % Difference(s) in the back calculated concentration obtained with fresh and stability samples for the following stability studies is within +15%. Stability of Lumefantrine and IS in Mobile Phase when placed in auto sampler. Stability of Lumefantrine in Biological matrix at ambient temperature. Stability of Lumefantrine in Biological matrix subjected to freeze-thaw cycles. Method has been validated for matrix affects i.e. no significant effect of different sources of matrix on accuracy of analytical method. Method is also validated to re-inject any sample whenever re-injection required. The bio analytical method validation report indicates the suitability of the method for analysis of subject samples.

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