AMERICAN JOURNAL OF PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

An Efficient RP-HPLC Method for the Simultaneous Quantitative Determination of Artemether and Lumefantrine In Human Plasma by using Dad Detection

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ABSTRACT

Artemether-lumefantrine (ART-LUME) off late has become the first-line treatment for uncomplicated malaria in many Sub-Saharan Africa, Asia and America. Vigorous monitoring of the therapeutic efficacy of this treatment is needed. This requires high-quality studies following standard protocols; ideally, such studies should incorporate measurement of drug levels in human plasma in biological matrices. A specific and reliable isocratic mode RP-HPLC method has been developed and validated for simultaneous determination of artemether (ART) in combination with lumefantrine (LUME) in human plasma using diode array detector (DAD) at 238 nm. The analyte was separated on NUCLEOSIC-CN Cyano coloumn (150 mm × 4.6 mm, particle size 5 µm) using a mobile phase consisting of acetonitrile and acidic buffer (adjusted to pH 2.5 with H_3PO_4 – 2 %) in the ratio of 37: 63 v/v and flow rate was 1 ml/min. The method is linear over a range of 100-1600 µg/ml ($r^2 \geq 0.999$) and 1-16µg/ml ($r^2 \geq 0.998$) for the assay of ART and LUME respectively. Itraconazole (ITZ) (10µg/ml) was used as internal standard. The retention times of ART and LUME was found to be 4.3 min and 14.3 respectively. Mean extraction recovery for ART and LUME were 87.3 % and 89.1 %, respectively. Inter and intraday coefficients of variation for ART and LUME were ≤ 10%. The lower limits of quantification for ART and LUME were 0.22 and 0.66 µg/ml, respectively. The results of the study showed that the proposed RP-HPLC validated method described is efficient and has the necessary accuracy and precision for the rapid quantitative simultaneous determination of ART and LUME in human plasma and is thus highly suitable for use in pharmacokinetic /bioavailability/bioequivalence studies in healthy human subjects.

Keywords: RP-HPLC method, Artemether, Lumefantrine, human plasma

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Received 12 January 2014, Accepted 29 January 2014

Please cite this article in press as: Sarma GS. *et al.*, An Efficient RP-HPLC Method for the Simultaneous Quantitative Determination of Artemether and Lumefantrine In Human Plasma by using Dad Detection. American Journal of PharmTech Research 2014.

INTRODUCTION

Malaria is caused by five species of parasitic protozoans of the genus *Plasmodium* that affect humans. *Plasmodium* parasites are transmitted to humans by the bite of infected female *Anopheles* mosquitoes. The World Malaria Report 2012 summarizes information received from 104 malaria-endemic countries. Children under five years of age and pregnant women are most severely affected by malaria, as their immune system is less able to fight *Plasmodium* infection. Malaria is currently endemic in 99 countries, causing an estimated 219 million cases and 660,000 deaths per year, with about 80 percent of cases and 90 percent of deaths occurring in Africa. Furthermore, insecticide resistance in malaria mosquito vectors is on the increase and artemisinin resistance has been identified in South-East Asia. Development of drug and insecticide resistance is causing concerns about losing key interventions in the fight against malaria. Artemisinin-based combination therapy (ACT) is increasingly being advocated as promising treatment. Artemether (ART) is a fast acting drug with short half life and reduces parasite biomass and quickly resolves clinical symptoms while, lumefantrine (LUME) acts slowly and has a longer half life which prevents recrudescence [1]. The registered fixed dose combination of ART (80 mg, Figure 1) and LUME (480 mg, Figure 2) (trade names Coartem and Riamet, Falcynate-LF, Lumitroy) is a fixed-dose combination of ACT based therapy indicated for the treatment of acute uncomplicated *Plasmodium falciparum* malaria.

Therefore therapeutic efficacy monitoring of the combination to provide advance warning even in case of minor changes in efficacy is needed. Pharmacokinetic and pharmacodynamic studies of Coartem have shown that plasma LUME concentration on day 7 is the principal determinant of treatment outcome, where values >500 ng/ml being associated with >90% cure rates and values < 280 ng/ml are predictive of treatment failures [2]. There are many methods available in literature till date pertaining to analysis of these drugs in plasma based on HPLC with electrochemical [3-5] or mass spectrometry detection [3]. Few methods are available to assay (ART) in pharmaceutical products [7, 8] The quantitative determination of LUME in plasma has been described using HPLC with UV detection [9-12]. RP-HPLC method for simultaneous estimation of ART and LUME has been reported by us in our previous paper [13]. However, there is no method reported to quantify simultaneously ART and LUME in human plasma using reverse phase HPLC (RP-HPLC) combined with a diode array detector (DAD) to be applicable in settings with limited facilities. The aim of the present study was therefore to develop and validate a comparatively simple and rapid RP- HPLC method for the quantitative determination of ART

and LUME in human plasma for application to a pharmacokinetic and bioavailability study in healthy human volunteers. The systematic and integrated assessment in disguise also provides sufficient evidence for the establishment of the quality standard.

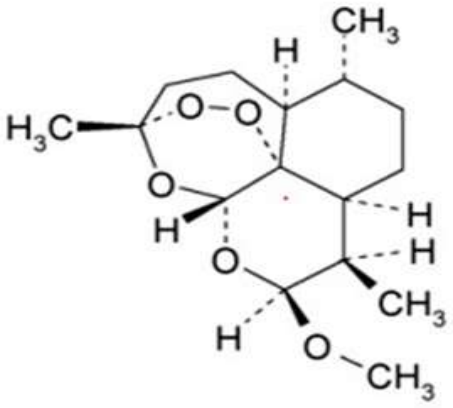

Figure 1: Artemether

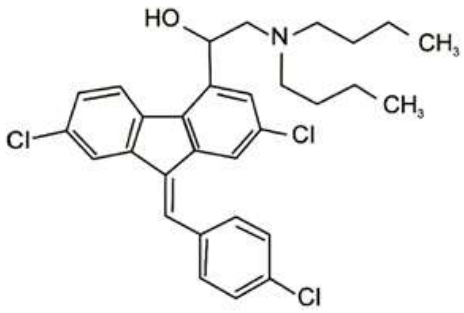

Figure 2:Lumefantrine

MATERIALS AND METHOD

Reagents and materials

ART and LUME reference standards were donated as gift samples by IPCA Pharmaceuticals ltd. (Mumbai, India) and Itraconazole (ITZ) used as an internal standard (IS) was obtained from the Yashick Pharmaceuticals Pvt Ltd (Thane, India). Market formulation Lumitroy (label claim of 80mg ART and 480mg LUME, combination) was purchased from Troikaa pharmaceuticals Ltd. Di-hydrogen ortho phosphate and 85% ortho-phosphoric acid (H_3PO_4) of analytical-reagent grade were purchased from Rankem (New Delhi, India) and were used without further purification. HPLC grade acetonitrile (ACN) was procured from the same supplier while, Ultra-pure water was obtained from a Milli-Q system (Millipore, USA). All other chemicals and solvents were of analytical reagents grade. All buffers and solutions were prepared with HPLC grade water.

HPLC instrumentation and chromatographic conditions

The HPLC system consisted of Waters 515 Series pumps combined with a PDA 2998 series photo diode array detector (DAD, wavelength range 190-800 nm) coupled to EMPOWER-2 data acquisition software (Waters, Milford, MA, USA). Chromatographic separation was achieved using a NUCLEOSIC-CN Cyano (5 µm, 150 mm × 4.6 mm i.d.) column maintained at 25 ˚C. UV detection was performed at 238 nm. The injection volume was 20 µl. An isocratic mobile phase containing acetonitrile-di-hydrogen ortho phosphate (adjusted to pH 2.5 with H_3PO_4 – 2 %) (37: 63, v/v) was used at a flow rate of 1.0 ml/min. The separation of ART and LUME was

evaluated in different proportions of these solvents and, for each condition, retention factor (k) and resolution (R) were calculated.

Preparation of calibration standards

Stock solution of reference standards of ART (10 mg/ml) and LUME (100 µg/ml) were freshly prepared in ACN (add 1ml of DMSO to increase solubility of LUME). Stock solution of ART and LUME was diluted with the same solvent to prepare working solutions to follow the linearity range (100- 1600 µg/ml for ART) and (1-16 µg/ml for LUME). A stock solution of the IS, ITZ, was likewise prepared at a concentration of 10 µg/ml. All stock solutions were stored in a refrigerator at 4±2 ˚C. Marketed formulation was prepared as similar to pure ART and LUME, then diluted accordingly after proper separation of excipients.

Plasma extraction procedure

A 100 µl of working stock solution of (ART and LUME) was spiked to blank plasma sample (100 µl). This spiked plasma sample was vortex mixed for 1 min. ACN (700 ml) was added to it, shaken well and separation was done by centrifugation (8000 rpm,10 min). From 1 ml of this supernatant taken, the residues were reconstituted in an ACN (100 µl). A 20 µl aliquot of final preparation was injected into the HPLC column. The same procedure was followed for the plasma without spiking the stock solution & IS equal volume (20 µL), of the samples that contain ART and LUME and ITZ in ACN were injected into the HPLC and the chromatograms were recorded. The responses (peak area) for the major peaks were measured and the quantity of ART, LUME and ITZ were calculated.

METHOD VALIDATION

Linearity

Standard solutions containing ART (10 mg/ml) and LUME (100 µg/ml) were prepared, in triplicate. Aliquots of these solutions were diluted in ACN and sonicated for 5 mins. Five point calibration curves were constructed in the range of 100- 1600 µg/ml for ART and 1-16 µg/ml for LUME, using least squares linear regression analyses.

Precision and Accuracy

The intra-day precision was evaluated by analyzing six sample solutions (n= 6), at lowest, intermediate, and highest quantification concentrations (LQC MQC and HQC) of the calibration curve, i.e. 100 µg/ml, 400 µg/ml, 1600 µg/ml for ART and 1µg/ml, 4µg/ml, and 16µg/ml for LUME. Similarly, the inter-day precision was evaluated in three consecutive days (6 injections / day, n= 18). The ART and LUME concentrations were determined and the relative standard deviations (R.S.D.) were calculated. Accuracy was calculated as the percent of ratio of ART and

LUME amount found to that of the actual of LQC, MQC and HQC. At each level, samples were prepared in triplicate and the recovery percentage was determined.

Extraction efficiency

Spiked biological samples were prepared in triplicate at three concentrations LQC, MQC, HQC of ART and LUME and 1 µg/mL of IS, and assayed as described above. The extraction efficiency was determined by comparing the peak areas measured after analysis of spiked plasma samples with those found after direct injection of non-biological (unextracted) samples or blank sample into the chromatographic system at the same concentration levels.

Detection and quantitation limits

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated by taking the AUC of standard and calculation is according to the 3.3s/m and 10s/m criterions, respectively, where s is the standard deviation of the AUC (n = 10) of the sample and m is the slope of the corresponding calibration curve.

Stability

Blank plasma was spiked with the known amount of ART and LUME to achieve the concentration of LQC, MQC and HQC of SC (n = 3) and stored at −4 °C. The stability of these samples was monitored for 15 days by comparing the results with fresh stock prepared on the day of analysis. Further, the freeze–thaw (−20 °C/room temperature) stability of the ART and LUME spiked plasma samples were determined for three cycles. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision. No internal standard was added prior to the analysis.

System Suitability Parameters

It was performed same as described for analytical method development in our earlier paper [13].

RESULTS AND DISCUSSION

Method development

A simple binary mobile phase of acetonitrile-di-hydrogen ortho phosphate (37: 63, v/v) were chosen based on our observation as reported in our previous paper [13] for the chromatographic separation of unextracted and extracted standard solutions of ART, LUME and IS dissolved in mobile phase. This ratio of mobile phase promoted an adequate separation (k = 2.26), and a short run time (ART = 4.3 min, LUME = 14.3 min), and so, this condition was adopted in subsequent analyses (Figure. 3). Using buffers with pH below the basic i.e. log P of ART and LUME, which were 3.48 [14] and 9.19 [15] respectively, instead of water as the aqueous component of the mobile

phase, improved the symmetry and sharpness of the peaks. ART shows UV absorption only in the initial wavelengths of the spectrum (200–220 nm), due to the absence of chromophores in its structure. Nevertheless, the artemether absorptivity is considerably low in this region, resulting in HPLC-UV methods with poor sensitivity and estimation in human plasma [16]. Hence, a RP-HPLC method with DAD detection was developed. This approach allowed an adequate ART and LUME detection and consequently quantitation at 238 nm.

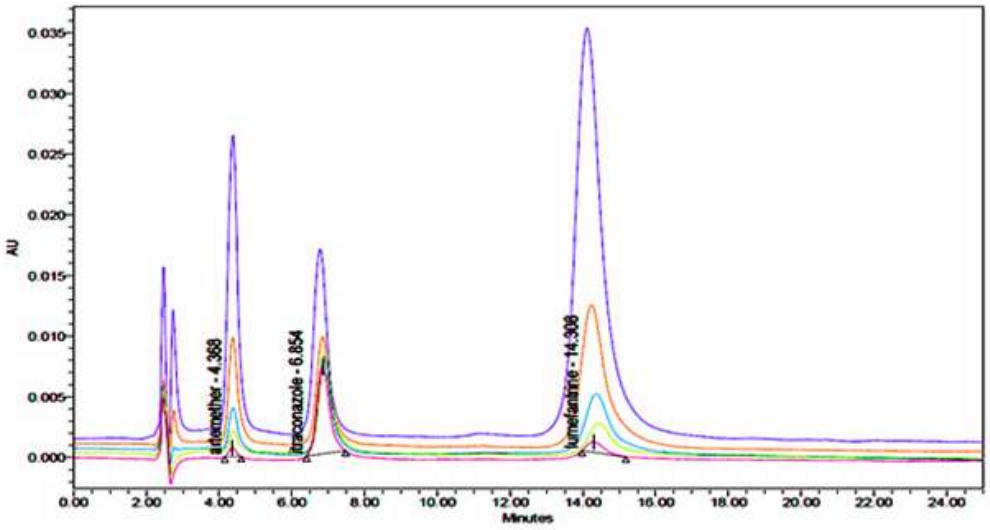

Figure 3: Overlay report of bioanalytical method for artemether and lumefantrine

Validation

Linearity

A linear correlation was found between the peak areas and the concentrations of ART and LUME, in the assayed range. The regression analysis data are presented in Table 1. The regression coefficients (r^2) obtained were higher than 0.99 for both compounds, which attest the linearity of the method.

Table 1: Calibration curve data for ART and LUME

Regression parameters	Artemether	Lumefantrine
Linearity range	100-1600µg/ml	1-16µg/ml
UV detection	238nm	238nm
Regression coefficient, r^2	0.999	0.998
Slope ± S.D*	0.00 ± 0.002	0.002 ± 0.001
Intercept ± S.D*	0.036 ± 1.23	0.227 ± 0.31
Relative standard error (%)	0.36	0.57
Limit of detection (LOD)	4.5 µg/ml	0.5
Limit of quantification (LOQ)	14.5µg/ml	1.4
Retention time	4.3 min	14.3 min
Internal standard	6.8 min	6.8 min

*Standard deviation

Detection and quantitation limits

According to the literature data of signal-to-noise ratio, ART and LUME presented the detection limit established was 5 µg/ml of ART and 3.75 µg/ml of LUME and the quantitation limit was 15 µg/ml of ART and 11.25 µg/ml of LUME [16]. The same compounds proportion found in the sample solutions injected onto the chromatograph was noted with an improvement in detection limit established which was 4.5 µg/ml of ART and 0.5 µg/ml of LUME and the quantitation limit was 14.5 µg/ml of ART and 1.4 µg/ml of LUME .

Accuracy and Precision

Accuracy data in the present study ranged from 94.3 to 98.5 % indicates that there was no interference of endogenous plasma components in simultaneous estimation of ART and LUME determination. Inter-day as well intra-day replicates of ART and LUME, gave % R.S.D. below 7.00 for ART and below 7.5 for LUME, (should be less than 15 % according to CDER guidelines for bio-analytical method validation), revealed that the proposed method is highly precise. Intra-day precision and inter-day variation of method were determined using three replicate injections of three concentration levels and analyzed on same day for three times and three different days. The results of mean content of accuracy, inter-day and intra-day precision are explained in Table 2 demonstrating the accuracy and precision of the method.

Table 2: Accuracy and precision determination of ART and LUME in human plasma

Levels	**Precision**				**Accuracy (%)**	
	Intra-day (% RSD) (n = 5)		**Inter-day (% RSD) (n = 18)**			
	ART	LUME	ART	LUME	ART	LUME
LQC	9.115	5.525	4.680	5.303	82.6	88.4
MQC	7.925	9.620	9.475	6.811	90.8	87.3
HQC	2.734	2.834	7.069	2.494	89.8	91.7
Mean	6.591	5.993	7.075	4.869	87.3	89.1

ART: artemether. LUME: lumefantrine. n = number. RSD: residual standard deviation.

Analysis of fixed dose combination tablets

Assay fixed dose combination tablets (Lumitroy) containing 80mg ART and 480mg LUME, combination were analyzed using the validated chromatographic RP-HPLC method and it was found to be accurate and reliable. The results obtained are presented in Table 4. All analyzed batches presented ART and LUME contents very close to the labelled amount. The selective and specific estimation of ART and LUME in the presence of excipients of tablet dosage form and percentage purity was found to be 105.2 % w/w for ART and 96.21% w/w for LUME. The results for the percentage purity are shown in Table 3.

Table 3: Contents assay of ART and LUME in the fixed dose combination tablets (n=9)

Sample tablet	Batch	Concentration (µg/ml)	Amount estimated (µg/ml)	AUC ratio	Content (w/w%) ± S.D
	A	400	386.72	0.2783	101.32 ± 0.028
		4	3.89	0.8601	98.43 ± 0.055
Lumitroy	B	400	382.33	0.2115	98.5 ± 0.021
		4	3.12	0.8657	101.69 ± 0.068
	C	400	385.21	0.3016	105.2 ± 0.029
		4	3.67	0.8573	96.21 ± 0.025

Stability

The plasma samples at low (LQC), medium (MQC) and high (HQC) concentrations were stored at −4 °C, the short term stability and freeze thaw stability were checked. The results of freeze thaw stability data are explained in Table 4 (a, b). All the samples were found to be stable as the % RSD was less than 15%.

Table 4 (a): Short Term Stability Data for Bio-analytical Method for ART and LUME

Storage conditions	QC Concentration (µg/ml)					
	LQC		MQC		HQC	
	ART	LUME	ART	LUME	ART	LUME
0 hr % RSD	4.818	6.227	9.595	4.480	3.082	3.127
6 hr% RSD	11.436	5.747	7.174	6.766	7.416	1.840
12 hr% RSD	5.676	6.113	6.915	6.927	3.171	2.198
18 hr% RSD	4.885	8.508	13.054	8.233	2.816	1.691
24 hr% RSD	14.593	5.369	9.185	7.050	4.121	2.206

Table 4(b): Freeze Thaw Stability Data for ART and LUME

Storage Cycle	Level – QC concentration (µg/ml)					
	LQC		MQC		HQC	
	ART	LUME	ART	LUME	ART	LUME
1st cycle	1.207	0.103	0.480	0.042	0.970	0.112
2nd cycle	1.192	0.100	6.887	0.289	0.977	0.251
3rd cycle	1.248	0.392	6.680	0.390	0.985	0.512

Table 5: System suitability parameters of bio-analytical method for ART and LUME

System suitability parameter	ART result	LUME result
No. Of Theoretical Plates (Efficiency)	2660.2	4539.9
Capacity Factor	3.2	4.7
Selectivity	1.2	2.6
Resolution	6.3	6.3
Tailing Factor	1.0	1.5
Asymmetric Factor	1.2	1.5

Suitability Parameters

The system suitability parameters such as asymmetric factor, tailing factor, theoretical plates and

plate numbers were measured. The values found for these parameters are described in Table 5 All the system suitability parameters found to be according to the acceptable limits of the bio-analytical methods suggesting the efficacy of the method in quantifying the ART and LUME in human plasma.

CONCLUSION

As previous reported by our study of HPLC determination of ART and LUME [13], this is also a relatively simple, accurate, precise and rapid but RP-HPLC method suitable for ART and LUME quanitation in use in human plasma for pharmacokinetic/bioavailability/bioequivalence studies has been developed and validated. This method has several advantages compared to previously reported methods such as the use of NUCLEOSIC-CN Cyano column which increases sensitivity of the method in low plasma concentrations and consequently decreased the volume of organic solvent (37, v/v) used in the mobile phase. The stability study of the plasma containing drug for short term and freeze thawing assessment makes the method rugged. This study was the first report of simultaneous determination of ART and LUME in fixed dose combination in human plasma. Based on the validation performed here, the extraction procedure, short retention times of ART and LUME (4.3 min and 14.3 respectively) makes this RP-HPLC method robust and amenable for adaptation for routine use in a developing country for control of malaria.

ACKNOWLEDGEMENTS

The authors extend their gratitude towards IPCA Pharmaceuticals ltd. (Mumbai, India) for generously donating artemether and lumefantrine for research. Yashick Pharmaceuticals Pvt Ltd (Thane, India) is acknowledged for their intraconazole sample. A special thanks to department of pharmaceutical analysis and quality assurance at I.S.F college of pharmacy for providing the experimental facilities for our research work and Shri. Parveen Garg for his valuable support.

REFERENCES

1. Martensson A, Stromberg J, Sisowath C, Msellem MI, Gil JP, Montgomery SM, et al. Efficacy of artesunate plus amodiaquine versus that of artemether-lumefantrine for the treatment of uncomplicated childhood Plasmodium falciparum malaria in Zanzibar, Tanzania. Clin. Infect. Dis. 2005; 41(8):1079-86.

2. Price R, Uhlemann AC, Van Vugt M, Brockman A, Hutagalung R, Nair S, et al. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multi-drug resistant falciparum malaria. Clin. Infect. Dis. 2006; 42(11):1570-7.

3. Navaratnam V, Mansor SM, Chin LK, Mordi MN, Asokan M, Nair NK. Determination of artemether and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies. J. Chromatogr. B. 1995; 669 (2): 289–294.
4. Karbwang J, Na-bangchang K, Molunto P, Banmairuroi V, Congpuong K. Determination of artemether and its major metabolite, dihydroartemisinin, in plasma using high-performance liquid chromatography with electrochemical detection. J. Chromatogr. B. 1997; 690 (1-2): 259–65.
5. Sandrenan N, Sioufi A, Godbillon J, Netter C, Donker M, Valkenburg V. J. Chromatogr. B. 1997; 691:145–153.
6. Souppart C, Gauducheau N, Sandrenan N, Richard F. Development and validation of a high-performance liquid chromatography–mass spectrometry assay for the determination of artemether and its metabolite dihydroartemisinin in human plasma**.** J. Chromatogr. B. 2002; 774 (2): 195–203.
7. Green MD, Mount DL, Wirtz RA. Authentication of artemether, artesunate and dihydroartemisinin antimalarial tablets using a simple colorimetric method. Trop. Med. Int. Health. 2001; 6 (12): 980–982.
8. Atemnkeng MA, De Cock K, Plaizier-Vercammen J. Quality control of active ingredients in artemisinin-derivative antimalarials within Kenya and DR Congo. Trop. Med. Int. Health. 2007; 12 (1): 68–74.
9. Zeng M, Lu Z, Yang S, Zhang M, Liao J, Liu S, Teng X. Determination of benflumetol in human plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. B. 1996; 681(2): 299–306.
10. Mansor SM, Navaratnam V, Yahaya N, Nair NK, Wernsdorfer WH, Degen PH. Determination of a new antimalarial drug, benflumetol, in blood plasma by high-performance liquid chromatography. J. Chromatogr. B. 1996; 682 (2): 321–325.
11. N. Lindegardh, A. Annerberg, D. Blessborn, Y. Bergqvist, N. Day, N.J. White, J. Pharm. Biomed. Anal. 2005; 37:1081–1088.
12. Annerberg A, Singtoroj T, Tipmanee P, White NJ, Day NPJ, Lindegardh N. High throughput assay for the determination of lumefantrine in plasma. J. Chromatogr. B. 2005; 822 (1-2): 330–333.
13. Vikas P, Ganti Subharmanya S, Seema R. RP-HPLC method for simultaneous estimation of artemether and lumefantrine. Int. J Uni Pharm and Life Sci. 2011; 1(1): 31-43.

14. Huang L, Lizak PS, Jayewardene AL, Marzan F, Lee M-NT, Aweeka FT. A modified method for determination of lumefantrine in human plasma by HPLC-UV and combination of protein precipitation and solid-phase extraction: application to a pharmacokinetic study. Anal Chem Insights. 2010; 5:15–23.
15. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use: Validation of analytical procedures: Text and methodology.ICH ed., volth edition. Geneva: Topic Q2 (R1); 2005.
16. Isabela da CC, Fernando Henrique AN, Gerson AP. Simultaneous determination of artemether and lumefantrine in fixed dose combination tablets by HPLC with UV detection. J of Pharma. and Bio. Anal. 2008;48(8): 951–54.