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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ARTEMETHER AND LUMEFANTRINE IN PURE AND PHARMACEUTICAL DOSAGE FORM USING RP-HPLC METHOD

(Pembangunan dan Validasi Kaedah Analisis bagi Anggaran Serentak Artemether dan Luefantrine dalam Keadaan Tulen dan Dos Farmaseutikal Mengunakan Kaedah KCPT-Fasa Terbalik)

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Abstract

A simple, rapid, precise and cost effective reversed phase-high performance liquid chromatography (RP-HPLC) method was developed for the simultaneous estimation of Artemether (AT) and Lumefantrine (LU) in pure drug and pharmaceutical dosage forms. The separation was carried out using BDS Hypersil C18 (150 × 4.6 mm i.d. 3 µm particle size) column, with mobile phase comprising of 0.01M tetra butyl ammonium hydrogen sulphate and acetonitrile in the ratio of 20 : 80 (v/v). The flow rate was 1.0ml/min and the detection was carried out using UV-visible detector at 222 nm. The method was validated by evaluation of different parameters such as accuracy, precision, linearity, ruggedness, and robustness, limit of detection (LOD) and limit of quantification (LOQ). The retention time were found to be 4.19 and 5.22 min for AT and LU, respectively. Correlation coefficient (r^2) of 0.999 for both over concentration range of 3.2-19.2µg/ml and 16-96µg/ml for AT and LU, respectively. Parameters like mobile phase ratio, wavelength, flow rate, etc. were deliberately varied. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed RP-HPLC method is robust. Intra and inter day precision reproducibility study was carried out and it was checked by determining precision on the same instrument, but by a different analyst. The percentage recovery for AT and LU were ranged between 99.18-100.19 and 99.96-100.07, respectively. The LOD for AT and LU were found to be 0.201 and 2.99 µg/ml and the LOQ were 0.609 and 9.086 µg/ml respectively. Method was found to be reproducible with relative standard deviation (RSD) for intra and inter day precision less than 2%.

Keywords: Artemisinin-Based Combination Therapy, Artemether, Lumefantrine, High Performance Liquid Chromatography (HPLC), Validation.

Abstrak

Satu kaedah kromatografi cecair berprestasi tinggi (HPLC) – fasa terbalik yang mudah, cepat, tepat dan kos efektif telah dibangunkan bagi anggaran serentak Artemether (AT) dan Lumefantrine (LU) di dalam ubat-ubatan bentuk tulen dan dos farmaseutikal. Pemisahan dilakukan menggunakan turus BDS Hypersil C18 (150 × 4.6 mm i.d 3 partikel saiz), dengan fasa bergerak terdiri daripada 0.01M tetra butyl ammonium hidrogen sulfat dan asetonitril pada nisbah 20:80 (v/v). Kadar aliran adalah 1.0 ml/min dan pengesanan dibuat menggunakan pengesan UV yang boleh diukur pada 222 nm. Kaedah divalidasi oleh ujian penilaian parameter yang berbeza seperti ketepatan, kejituan, linear, kekasapan dan keteguhan, had pengesanan (LOQ) dan had kuantifikasi (LOQ). Masa pengekalan didapati pada minit 4.19 dan 5.22 masing – masing untuk AT dan LU. Pekali korelasi (r^2) 0.999 kedua-duanya pada julat kepekatan masing – masing 3.2 - 19.2µg/ml dan 16 - 96µg/ml bagi AT dan LU. Parameter seperti nisbah fasa bergerak, panjang gelombang, kadar aliran, dan lain-lain telah dijalankan. Diperhatikan bahawa tiada terdapat sebarang perubahan ketara bagi kromotogram, yang membuktikan bahawa kaedah fasa terbalik-KPCT ini adalah baik kadar kekasapannya. Ujian kebolehulangan inter dan intra hari telah dijalankan dan ia adalah ketepatan telah ditentukan pada instrumen yang sama oleh penganalisa berbeza. Perolehan semula bagi AT dan LU adalah masing – masing di antara 99.18 -

100.19 dan 99.96 - 100.07. Had pengesanan, LOD bagi AT dan LU didapati pada 0.201 dan 2.99 µg/ml dan LOQ masing-masing adalah 0.609 dan 9.086 µg/ml. Kaedah ini didapati nilai kebolehulangannya dengan kejituan sisihan piawai relatif (RSD) bagi intra dan inter – hari kurang daripada 2%.

Kata kunci: Kombinasi terapi berasaskan-artemisinin, Artemether, Lumefantrine, kromatografi cecair prestasi tinggi (KCPT), Validasi

Introduction

Due to the widespread resistance of *Plasmodium falciparum* to conventional antimalarial drugs, many countries are facing problems regarding the treatment of uncomplicated malaria [1]. The main therapy now recommended by the World Health Organization (WHO) is artemisinin-based combination therapy (ACT), a combination of an artemisinin derivative and another structurally-unrelated and slowly-eliminated antimalarial lumefantrine [2]. The advantages of ACT relates to the properties of artemisinin compounds, which include rapid reduction of the parasite biomass with fast resolution of clinical symptoms, effectiveness against multidrug-resistant *falciparum* malaria, resistance not being documented yet, and a good safety profile. They also reduce gametocyte carriage, which in some settings may lower malaria transmission [3]. Artemether is chemically (3R,5aS,- 6R,8aS,9R,10S,12R,12aR)-Decahydro-10-methoxy- 3,6,9- trimethyl- 3,12-epoxy-12H-pyrano [4,3-j]-1,2- benzodioxepin[4] and is used as antimalarial agent. Lumefantrine is chemically 2, 7-Dichloro-9-[(4- chlorophenyl) methylene]-α-[(dibutylamino) methyl] - 9H-fluorene-4-methanol [5] and is used in the treatment of uncomplicated *falciparum* malaria.

Artemether-lumefantrine (AT-LU) (Figure 1) is the most common ACT used in malaria endemic areas. The rationale is that Artemether will rapidly reduce parasitaemia, resulting in symptomatic relief, and Lumefantrine will eliminate the remaining parasites [6]. WHO recommends this combination as first line therapy for *falciparum* malaria in endemic areas [7]. Increasing use of (AT-LU) combination as an effective treatment for malaria demands the need of analytical methods to simultaneously quantify these drugs in tablets in order to evaluate its quality. Some of the studies have described the analysis of AT in plasma, based on HPLC with electrochemical [8, 10] or mass spectrometry detection [11]. Few methods are available to assay AT in pharmaceutical products [12, 13]. The quantitative determination of lumefantrine in plasma has been described using HPLC with UV detection [14, 15]. However, there is lack of methods regarding the simultaneous quantitation of AT and LU. Hence, the aim of this study was to develop and validate a reversed phase-high performance liquid chromatography (RP-HPLC) method, using UV detection, to simultaneously quantify Artemether and Lumefantrine in fixed dose combination tablets. The structure of Artemether and Lumefantrine is as shown below in Figure 1.

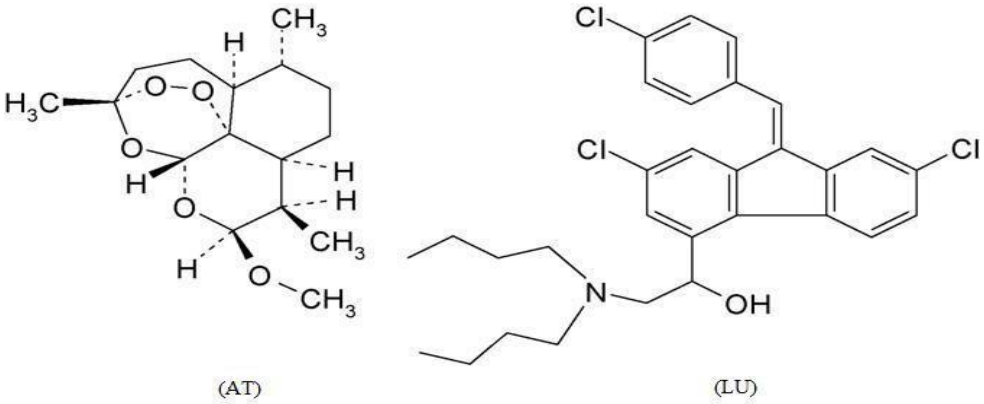

Figure 1. Structure of Artemether and Lumefantrine

Materials and Methods

Reagents

AT and LU reference standards were obtained as a gift sample from Ipca Pvt ltd. Mumbai. Market formulation LUMERAX-80 (AT and LU, combination) from Ipca Laboratories Ltd, Ratlam with a label claim of 80mg AT and 480mg LU, was purchased. Tetra butyl ammonium hydrogen sulphate of analytical-reagent grade was purchased from Leonil Chemicals Pvt Ltd, Bangalore, India. HPLC grade acetonitrile (ACN) and water were purchased from Merck Specialities Pvt Ltd, Mumbai. All buffers and solutions were prepared with HPLC grade water.

Instrumental

The separation was carried out using A HPLC unit that consisted of a LC-20AT Shimadzu pumps combined with a SPD-20A Prominence UV-VIS detector. The column used was BDS Hypersil C-18 (150×4.6mm i.d.; 3µm particle size). Analyte weighing was done on a microbalance, Shimadzu AY220. All mobile phase solutions were degassed ultrasonically by "Fast Clean" sonicator before use. The HPLC system was controlled by a PC workstation using Spinchrom software.

Chromatographic conditions

The HPLC was operated under isocratic elution with acetonitrile-0.01M tetrabutylammonium hydrogen sulphate buffer (80:20, v/v) as a mobile phase at a flow rate of 1.0 ml/min. The mobile phase was premixed, filtered through a 0.45 µm membrane filter to remove any particulate matter and degassed by sonication before use. The separation of AT and LU was good enough (Figure 2) and further it was free from any interference at 222 nm. Hence, the eluted peaks were detected at 222 nm for both, AT and LU. Moreover, the effects at different levels of all these factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, separation factor, asymmetry, and Height Equivalent to Theoretical Plate (HETP) etc.

Preparation of buffer

Dissolve 0.33954 gm of tetra butyl ammonium hydrogen sulphate in small amount of distilled water, transferred into 100ml volumetric flask and made up to the final volume with HPLC grade water.

Preparation of mobile phase

200ml (20%) of the above buffer was mixed with 800 ml of acetonitrile (80%). Mobile phase will be filtered first using 0.45 µm membrane filter and then degassed in an ultrasonic water bath for 5 minutes prior to use.

Diluent Preparation

Mobile phase [0.01M tetra butyl ammonium hydrogen sulphate & acetonitrile (20:80%v/v)] used as a diluent.

Standard solution preparation

Accurately weighed and transferred 4mg of AT reference standard and 24mg of LU reference standard to 25ml volumetric flask, added appropriate quantity of diluent and sonicated to dissolve it completely and the volume was made up to the mark with the same diluent, to obtain a solution of 160µg/ml of AT and 960µg/ml of LU, resultant solution was ultra sonicated for 5min and filtered through 0.45µ filter paper.

Sample solution preparation

Five tablets of AT and LU were weighed and finely powdered. A quantity equivalent to 4mg of AT and 24mg of LU was transferred into 25 ml volumetric flak and appropriate amount of diluent was added. The contents were sonicated to dissolve AT and LU completely and the volume was made up to the mark with diluent and filtered through 0.45µm membrane filter.

Results and Discussion

Optimization of chromatographic conditions

The mobile phase conditions, such as the type and composition of the organic modifiers significantly affect the chromatographic separation, therefore before selecting the conditions for optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, mobile phase compositions and flow rate to check the retention time, shape, resolution, and other chromatographic parameters. The chromatographic parameters were initially evaluated using a inertsil ODS C18 (150x4.6; 5µ)

column and a mobile phase composed of acetonitrile and water (50:50), since no peaks were obtained for both the drugs, thus water was replaced by a tetra butyl ammonium hydrogen sulphate (0.01 M). Mixture of acetonitrile and tetra butyl ammonium hydrogen sulphate buffer in different ratios (60:40, 70:30 and 80:20) was tried as mobile phase. From those experiments the mobile phase combination of acetonitrile and tetra butyl ammonium hydrogen sulphate buffer in the ratio of 80:20 was found to be more appropriate.

The retention time obtained for AT and LU is 4.19 and 5.22 respectively thus indicating the developed method is rapid compared to T.M Kalyankar *et al* method having retention time of 6.15 min and 11.31min, for AT and LU respectively [16], The retention times of Artemether and Lumefantrine were 13.888 min and 7.207 min respectively in Sridhar B. *et al* method [17]. The retention times were 13.887 and 7.218 mins for Artemether and Lumefantrine, respectively in Sunil J. *et al* method [18].Thus proving the developed method to be more cost effective, rapid and precise. A typical chromatogram of Artemether and Lumefantrine is as shown in Figure 2.

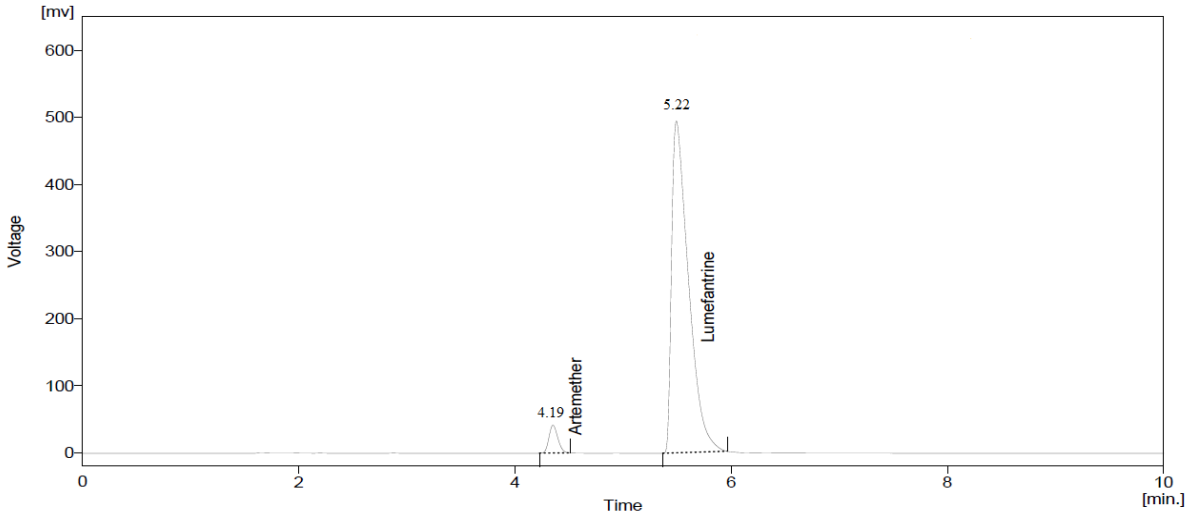

Figure 2. A typical chromatogram of Artemether and Lumefantrine

Method Validation

Once the chromatographic method was developed and optimized, it must be validated. After optimization of the chromatographic conditions, the parameters of linearity, precision, accuracy, ruggedness, robustness, limit of detection and limit of quantitation were evaluated to validate the process. The validation of an analytical method verifies that the characteristics of the method satisfy the requirements of the application domain. The proposed method was validated as per ICH Guidelines [19].

System suitability

System suitability tests are an integral part of chromatographic method. They were used to verify the adequate reproducibility of chromatographic system for analysis. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared standard stock solution of Artemether and Lumefantrine and the parameters like column efficiency, resolution, peak area and tailing factor of the peaks were calculated. For all the samples analyzed, the efficiency, %RSD and USP tailing factor were found to be $\geq$ 2000 theoretical plates, $\leq$ 2% and $\leq$ 2 respectively. System suitability test results are as shown in Table 1.

Table 1. System suitability test for Artemether and Lumefantrine

Artemether				Lumefantrine			
Area (mV)	**RT**	**TF**	**TP**	**Area (mV)**	**RT**	**TF**	**TP**
1448.917	4.190	1.258	5755	3994.438	5.227	1.538	5037

Linearity

The standard solutions containing 3.2 µg/ml to 19.2 µg/ml of AT and 19.2 µg/ml to 115.2µg/ml of LU in each linearity level were prepared and injected. In the simultaneous determination, the calibration curves were found to be linear for both the analytes in the mentioned concentrations. The analytical curve with peak area versus concentration was plotted and the obtained data were subjected to regression analysis. The coefficient of correlation (r^2) was found to be 0.999 for AT and LU. Calibration curve of Artemether and Lumefantrine at 222 nm is shown in Figure 3 and 4 respectively and linearity result for Artemether and Lumefantrine is as shown in Table 2 and Table 3 respectively.

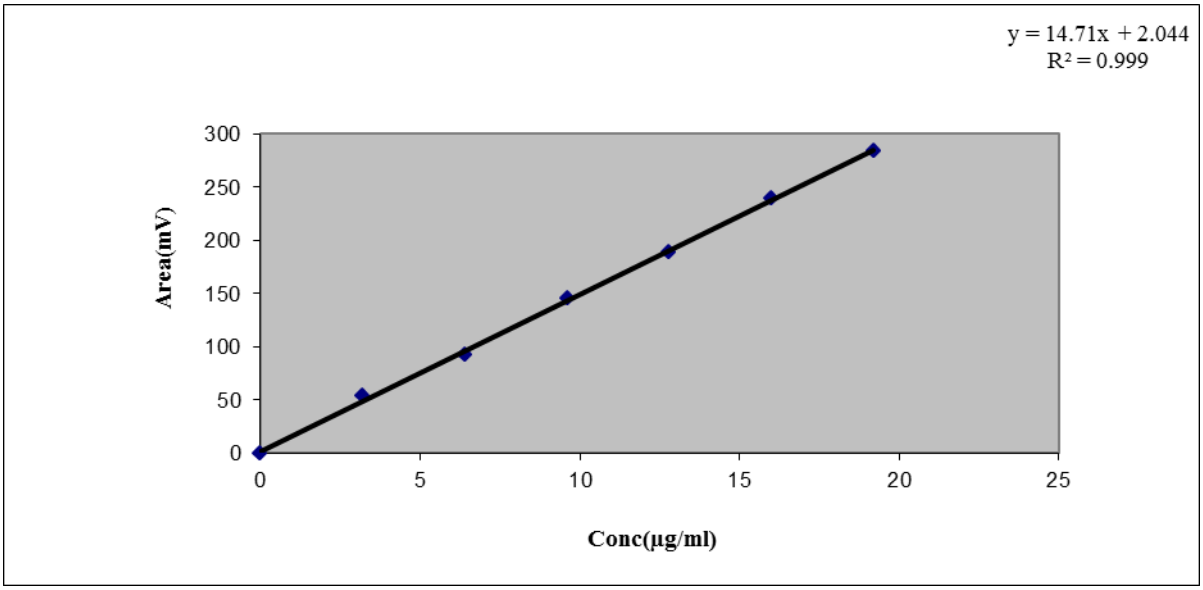

Figure 3. Calibration curve of Artemether at 222 nm

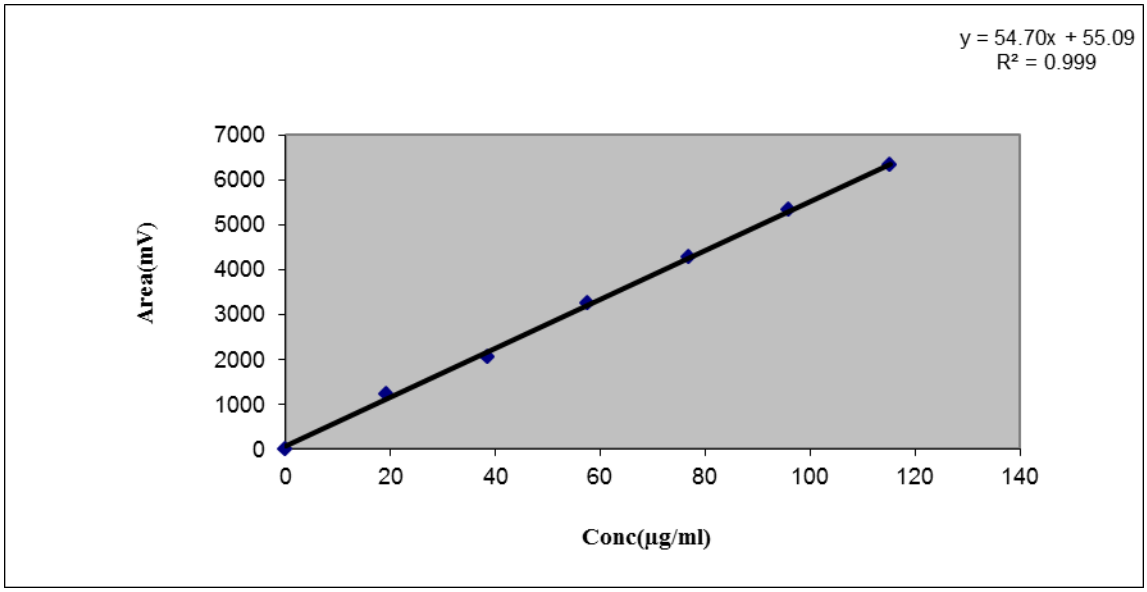

Figure 4. Calibration curve of Lumefantrine at 222 nm

Table 2. Linearity result for Artemether

Sample No.	Concentration(µg/ml)	Area(mV)
1	19.2	1222.24
2	38.4	2048.86
3	57.6	3236.65
4	76.8	4283.97
5	96.0	5331.53
6	115.2	6317.87

Table 3. Linearity result for Lumefantrine

Sample No.	Concentration (µg/ml)	Area(mV)
1	3.2	53.537
2	6.4	92.728
3	9.6	144.805
4	12.8	189.063
5	16.0	239.394
6	19.2	283.343

Precision

The precision of the method was demonstrated by inter-day and intra-day variation studies. The intra-day precision was evaluated by injecting six times each of the standard solution i.e.16µg/ml of AT and 96µg/ml of LU. Similarly, the inter-day precision was evaluated in five consecutive days. The AT and LU concentrations were determined and the relative standard deviations (R.S.D.) were calculated. Intra-day and Inter-day precision result for Artemether and Lumefantrine is as shown in Table 4 and Table 5 respectively.

Table 4. Intra-day precision result for Artemether and Lumefantrine

Injection No.	**Artemether**		**Lumefantrine**	
	Area(mV)	**RT**	**Area(mV)**	**RT**
1	238.008	4.343	5372.668	5.490
2	238.749	4.360	5373.642	5.513
3	241.630	4.357	5373.611	5.510
4	240.339	4.340	5367.166	5.487
5	238.402	4.350	5365.531	5.493
Average	239.426	4.35	5370.524	5.499
SD	1.517	0.0086	3.874	0.012
% RSD	0.630	0.190	0.072	0.210

Table 5. Inter-day precision result for Artemether and Lumefantrine

Injection No.	**Artemether**		**Lumefantrine**	
	Area(mV)	**RT**	**Area(mV)**	**RT**
1	241.313	4.353	5477.071	5.487
2	241.049	4.33	5376.734	5.46
3	239.156	4.347	5374.384	5.487
4	240.222	4.353	5361.259	5.493
5	239.739	4.343	5357.487	5.487
Average	240.296	4.345	5389.387	5.483
SD	0.897	0.009	49.705	0.013
%RSD	0.370	0.220	0.920	0.240

Accuracy

For studying the accuracy of the proposed analytical method, and for detecting the interference from excipients used in the dosage forms, recovery experiments were carried out by the standard addition method. Known concentration of working standard was added to the fixed concentration of the pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of working standard. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate. At 80%, 100% and 120% level and the percentage recovery was calculated. Percent recovery indicates that the method was accurate. Recovery results for Artemether and Lumefantrine is as shown in Table 6 and Table 7 respectively.

Table 6. Recovery results for Artemether

Accuracy level (%)	Amount added in µg	Area	Amount recovered in µg (Average)	% Recovery (Average)	SD	%RSD
80	14.4	218.152	14.29	99.25	1.511	0.697
100	17.6	264.151	17.46	99.18	0.815	0.308
120	20.8	316.796	20.84	100.19	1.732	0.548

Table 7. Recovery results for Lumefantrine

Accuracy level	Amount added in µg	Area	Amount recovered in µg (Average)	% Recovery (Average)	SD	%RSD
80	86.4	4831.711	86.46	100.07	3.517	0.072
100	105.6	5908.646	105.62	100.02	3.360	0.056
120	124.8	6978.117	124.75	99.96	5.604	0.080

Sensitivity

LOD and LOQ were estimated from the signal-to-noise ratio. The LOD for AT and LU were found to be 0.201 and 2.99 µg/ml and the LOQ were 0.609 and 9.086 µg/ml respectively.

Ruggedness (Reproducibility)

In addition to intra and inter day precision reproducibility study was also carried out and it was checked by determining precision on the same instrument, but by a different analyst. Results for Ruggedness are shown in Table 8.

Table 8. Ruggedness studies of Artemether and Lumefantrine

	Artemether		**Lumefantrine**	
Sample No.	**Analyst 1 Area(mV)**	**Analyst 2 Area(mV)**	**Analyst 1 Area(mV)**	**Analyst 2 Area(mV)**
1	238.008	238.749	5372.668	5373.642
2	237.894	238.344	5373.374	5299.893
3	238.016	237.995	5354.612	5362.331
Average	237.972	238.362	5366.884	5345.288
SD	0.068	0.377	10.634	39.718
% RSD	0.028	0.158	0.198	0.743

Robustness

To evaluate robustness of the developed method, few parameters like mobile phase ratio, wavelength, flow rate, etc. were deliberately varied. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed RP-HPLC method is robust. Robustness results for Artemether and Lumefantrine is as shown in Table 9 and Table 10 respectively.

Table 9. Robustness results for Artemether

Condition	**Modification**	**Mean Area ± SD***	**% RSD**
Flow Rate (ml/min)	0.9	269.590 ± 1.35	0.50
	1.0	240.079 ± 0.968	0.403
	1.1	219.149 ± 0.543	0.247
Wavelength (nm)	220	262.031 ± 0.632	0.241
	222	240.079 ± 0.968	0.403
	224	217.075 ± 0.725	0.333

* Average of three determinations.

Table 10. Robustness results for Lumefantrine

Condition	Modification	Mean Area ± SD*	% RSD
Flow Rate (ml/min)	0.9	6080.142 ± 82.522	1.357
	1.0	5364.53 ± 46.67	0.869
	1.1	4801.298 ± 6.624	0.137
Wavelength (nm)	220	5349.852 ± 4.492	0.083
	222	5364.53 ± 46.67	1.357
	224	5704.685 ± 11.54	0.202

*Average of three determinations.

Conclusion

Considering the increasing use of ACT to treat malaria in endemic areas, the availability of simple and rapid analytical method is essential to evaluate the quality of formulas being used currently. From the present study it can be concluded that the optimized and validated RP-HPLC method was simple, sensitive, precise, accurate and reproducible, hence it can be used in routine analysis for the simultaneous determination of Artemether and Lumefantrine in bulk as well as in pharmaceutical preparations.

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References

1. Martensson, J., Stromberg, C., Sisowath, M. I., Msellem, J. P., Gil, S. M., Montgomery, P., Olliaro, A. S., Ali. & A., (2005). 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*. 41: 1079 – 1086.
2. WHO (2001). Antimalarial *Drug Combination Therapy. Report of a WHO Technical Consultation, 4-5 April,* (WHO/CDS/RBM/2001.35). Geneva: WHO.
3. Nosten, F. & White, N.J., (2007). Artemisinin based combination treatment of falciparum malaria, Am. *J. Trop. Med. Hyg*. 77: 181 – 192.
4. Martindale: The complete drug reference 36th edition, Pharmaceutical press, Lambeth High Street, London, (2009); 598 - 599.
5. Martindale: The complete drug reference 36th edition, Pharmaceutical press, Lambeth High Street, London, (2009); 605.
6. Omari, A.A., Gamble, C. & Garner, P., (2004). Artemether-lumefantrine for uncomplicated malaria: a systematic review, *Trop. Med. Int. Health*. 9: 192 – 199.
7. World Health Organization, WHO Expert Committee on Malaria, 20th Report, WHO Technical Report Series – 892, Geneva, 2000; 71.
8. Navaratnam, V., Mansor, S.M., Chin, L.K., Mordi, M.N., Asokan, M., Nair & N.K., (1995). Determination of artemether and dihydroartemisinin in blood plasma by high-performance liquid chromatography for application in clinical pharmacological studies, *J. Chromatogr. B*, 669: 289 – 294.

9. Karbwang, J., Na-bangchang, K., Molunto, P., Banmairuroi, V. & Congpuong, K., (1997). Determination of artemether and its major metabolite, dihydroartemisinin, in plasma using high-performance liquid chromatography with electrochemical detection, *J. Chromatogr. B*, 690: 259 – 265.
10. Sandrenan, A., Sioufi, J., Godbillon, C., Netter, M., Donker, V. & Valkenburg., (1997). Determination of artemether and its metabolite, dihydroartemisinin, in plasma by high-performance liquid chromatography and electrochemical detection in the reductive mode, *J. Chromatogr. B*, 691: 145 – 153.
11. Souppart, C., Gauducheau, N., Sandrenan, N. & Richard, F., (2002). 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*, 774: 195 – 203.
12. Green, M.D., Mount, D.L. & Wirtz, R.A., (2001). Authentication of artemether, artesunate and dihydroartemisinin antimalarial tablets using a simple colorimetric method, *Trop. Med. Int. Health*, 6 : 980 – 982.
13. Atemnkeng, M.A., De Cock, K. & Plaizier-Vercammen, J., (2007). Quality control of active ingredients in artemisinin derivative antimalarials within Kenya and DR Congo, *Trop. Med. Int. Health*, 12: 68 – 74.
14. Mansor, S.M., Navaratnam, V., Yahaya, N., Nair, N.K., Wernsdorfer, W.H. & Degen, P.H., (1996). Determination of a new antimalarial drug, benflumetol, in blood plasma by high-performance liquid chromatography, *J. Chromatogr*. B, 682: 321 – 325.
15. César I, Nogueira F. & Pianetti G, (2008). Simultaneous determination of artemether and lumefantrine in fixed dose combination tablets by HPLC with UV detection, *J. of Pharm. Biomed. Analysis*. 45: 951 - 954.
16. Kalyankar T.M. & Kakde R.B. (2011) Reversed-phase liquid chromatographic method for simultaneous determination of artemether and lumefantrine in pharmaceutical preparation. *International Journal of ChemTech Research*, 3(3): 1722 - 1727.
17. Sridhar B., Rao K.H., Srinivas T.V.S., Madhuri V.S., Madhuri K. & Rao J.V.L.N.S.(2010). A validated reverse phase hplc method for the simultaneous estimation of artemether and lumefantrine in pharmaceutical dosage forms. *An International Journal of Advances In Pharmaceutical Sciences* 1(1): 95 - 99.
18. Sunil J., Nath M.S., .M & Moorthy U.S. (2010) HPLC method development and validation for simultaneous estimation of artemether and lumefantrine in pharmaceutical dosage forms. *International Journal of Pharmacy and Pharmaceutical Sciences.* 2(4): 93 - 96.
19. CMPM/ICH/381/95, ICH Topic Q2B Validation of Analytical Procedures: Methodology. Step 4 consensus guideline, (1996); Nov: 1-9