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Development and validation of HPLC-UV method for quantification of artemether in plasma

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Abstract
The pharmacokinetic knowledge of drug is vital for new drug development and to ensure the improvement in its bioavailability. The artemisinin derivative, artemether is a potent and rapidly acting antimalarial drug recommended by WHO. The objective of the study was to develop a simple and reliable bioanalytical method for the analysis of artemether in plasma.
An HPLC-UV method was developed and validated for the quantification of artemether in plasma of mice using artemisinin as internal standard. Artemether was extracted from plasma using protein precipitation method with acetonitrile followed by solid-phase extraction with hypersep C8 cartridges. Artemether and artemisinin were eluted at 8 and 5.3 minutes respectively. The method was found linear over a range of 20-200 µg/ml. The precision was exemplified by relative standard deviation of 1.8021%. System suitability parameters were within the acceptance limits and ideal for the chromatographed sample. The developed bioanalytical method was found précise, easy and reliable.

Keywords: HPLC-UV method, artemether, plasma

Introduction
Malaria is one of the life threatening infection among various other parasitic infections. Currently in most of the countries although uncomplicated malaria [1] is almost eradicated, the most severe parasite falciparum still remains a big challenge for researchers. Additionally antimalarial drug resistance is the emerging crisis observed worldwide and needs to be controlled with appropriate necessary measures. Among the artemisinin derivatives artemether is the widely used for the treatment of uncomplicated falciparum malaria and is recommended by FDA as first -line - treatment for the disease. The WHO recommended medicine, artemether is also a potent and rapidly acting antimalarial agent and is very well known for the treatment of severe multiresistant malaria. Artemether is effective against *P. vivax*, chloroquine-sensitive, chloroquine-resistant strains and cerebral malaria [2, 3]. Artemether shows rapid clearance of parasetemia and speedily resolves the disease symptoms observed in drug resistant malaria and gametocyte stage of parasite [4]. However artemether have short half-life of 2-3h which leads to frequent dosing and increase the risk of drug toxicity [5, 6] On the other hand poor aqueous solubility of artemether leads to lower bioavailability. Presently artemether is formulated in various novel drug delivery forms like solid lipid nanoparticles, nanoemulsions, NLC and SMEEDS, which are found to enhance bioavailability of the drug [5, 6, 7] A simple and easy bioanalytical technique can easily determine the blood concentration of artemether and thus confirms its improved bioavailability. Bioanalysis is an extremely important aspect of drug research. For the development of effective and new antimalarial formulation, it is essential to perform pharmacokinetic studies. The objective of the present study was to develop and validate a simple and reliable bioanalytical method for the analysis of artemether in plasma.

Materials and Methods
Chemicals and Reagents
Analytical pure sample of artemether and internal standard artemisinin with purities greater

than 99% were obtained as gift sample from Themis labs, Mumbai, India. Acetonitrile (HPLC grade), water (HPLC grade), 0.2µm nylon membrane filters were purchased from whatman ®. Drug free animal plasma was collected from retro orbital plexus of mice in tubes containing EDTA. All the excipients and reagents were used as received. Animal experiments were carried out according to the CPCSEA (Committee for the purpose of the control and supervision on experiments on animals) guidelines CUSCP/IAEC/018/2011-2012

Instrument

HPLC analysis was performed on Agilent Technologies liquid chromatography comprising a quaternary pump with PDA detector and C18 column Princeton SPHER ODS5 (250 X 4.0 mm; 5 µ). Autosampler with 20 µl sample loop was equipped with HPLC system. The HPLC system was controlled with "Chemstation" software. An electronic analytical weighing balance (0.1 mg Mettler Toledo), a sonicator (Enertech electronics PVT.LTD) were used in this study.

Method

Selection of wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrum in the range of 200-400 nm for artemether. Suitable wavelength selected was 210 nm (Figure 1).

Chromatographic conditions

The developed method uses a C 18 column, (250 X 4.0 mm; 5 µ), mobile phase consisting of ACN: water in the proportion of 70:30 v/v. The mobile phase was set at a flow rate of 1.0 ml/minutes and the volume injected was 20 µl for every injection [11, 12]. The detection wavelength was set at 210 nm. The substance was quantified using its peak area ratio of artemether to artemisinin (Internal standard). Prior to injecting solutions, the column was equilibrated for at least 30 minutes with the mobile phase flowing through the system. Each solution was injected in triplicate and the relative standard deviation (R.S.D.) was measured.

Mobile phase preparation

The mobile phase was prepared by mixing acetonitrile and water in the ratio of 70:30 v/v and later it was sonicated for 10 minutes for the removal of air bubbles.

Preparation of stock and working standard solution

50 mg of artemether was accurately weighed and taken in 100 ml clean and dry volumetric flask containing 50 ml of ACN and then sonicated for two minutes to dissolve. Later the solution was made up to the mark using the mobile phase. This is considered as stock standard solution (500 µg/ml). From the stock solution, 1ml was pipetted out and the volume made up to 10 ml using the mobile phase to get a concentration of (50 µg/ml).

Preparation of stock and working sample solution

The protein precipitation method was used for extraction of artemether from plasma using acetonitrile as protein precipitant [13, 14]. 200 µL of blank plasma was spiked with 100 µL of standard artemether and 100 µL of internal standard from 500 µg/mL dilution of artemether and artemisinin respectively. This spiked plasma was vortexed for 2 minutes and further diluted to 1.0 mL with acetonitrile. The mixture was further vortexed for 4 minutes and centrifuged at 3000 rpm for 10 minutes. After centrifugation, 100 µL of the supernatant was collected. A 20.0 µL aliquot of supernatant was injected into the HPLC system.

Result and Discussion

Method development

A bioanalytical HPLC method was developed by considering system suitability parameters i.e. tailing factor (T), number of therotical plates (N), run time and the cost effectiveness. Figures 1 and 2 represent the chromatogram of blank solution and the standard solution (50 µg/ml.) respectively. A sharp peak of internal standard artemisinin was observed at 5 minutes as shown in figure 3. The optimized method developed resulted in the elution of artemether at 8 minutes and artemisinin at 5 minutes (figure 4). The total run time is 15 minutes. System suitability tests are essential part of method development and are used to ensure satisfactory performance of the chromatographic system [11] Retention time (Rt), number of theoretical plates (N), and peak asymmetric factor was evaluated for six replicate injections of the standard at working concentration. The results are given in table 1.

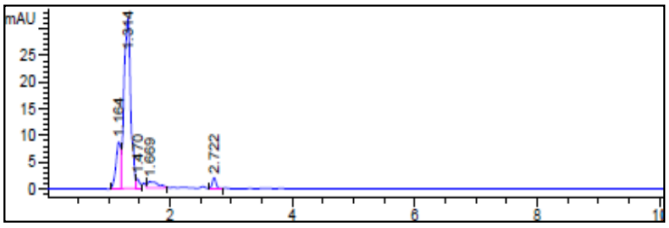

Fig1: Typical chromatogram of blank solution

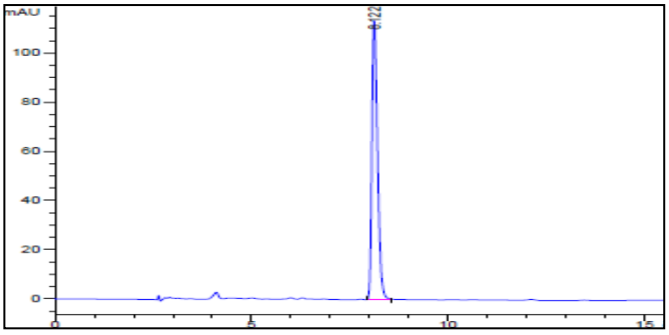

Fig 2: Typical chromatogram of standard solution (artemether)

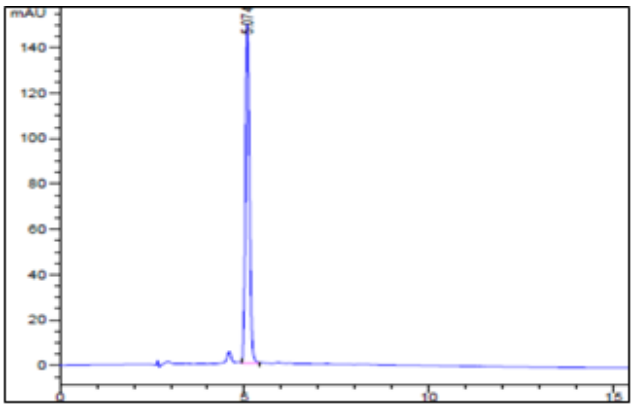

Fig 3: Typical chromatogram of internal standard (artemisinin)

Table 1: System suitability studies

Parameters	Artemether	Artemisinin
Retention time (minutes)	8.35	5.074
Number of theoretical plates	4397	2784
Tailing factor	0.91	0.839

In order to check the applicability of the developed method to a bioanalytical method, artemether was chromatographed at working concentration (50 µg/ml) as shown in figure 4. The peak was identified by comparing the retention time of sample peak with the standard drug (figure 2). System suitability parameters were within the acceptance limits, ideal for the chromatographed sample.

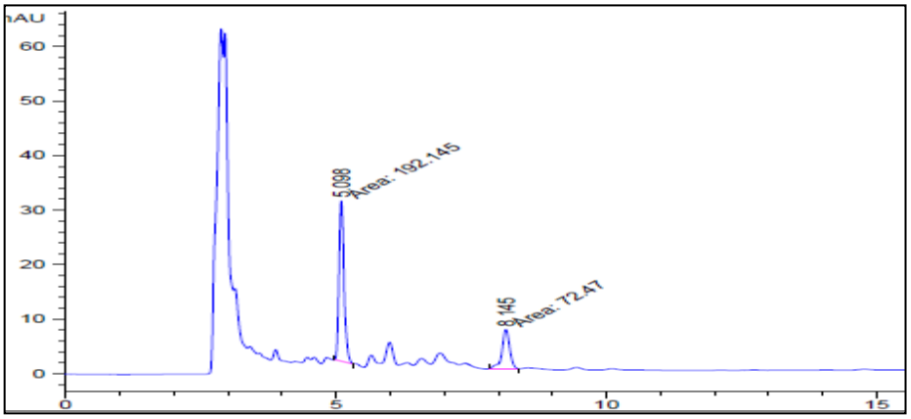

Fig 4: Typical chromatogram of plasma spiked with drug and internal standard

Method validation

Validation of the analytical method is the process that is established by laboratory studies in which the performance characteristics of the method meets the requirements for the intended analytical application. An isocratic HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines [15] for validation of analytical procedures. The method was validated in terms of parameters like system suitability, selectivity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantification (LOQ) [15].

Specificity

Figures (1-4) for blank, standard drug solution, internal standard and plasma sample chromatogram reveal that the peaks obtained in the standard solution and the sample solution at working concentrations are only because of the drug as blank has no peak at the retention time of artemether. Accordingly it can be concluded that, the method developed is said to be specific.

Precision

Table 2: System precision results

Injection number	Artemether	
(n)	Rt (minutes)	Peak Area
1	5.098	192.144
2	5.012	190.22
3	5.03	185.28
4	5.12	187.41
5	5.16	193.21
Average		189.664
SD		3.2812
% RSD		1.730

Table 3: Intra- day precision results

n	Artemether
	% Assay
1	99.56
2	96.60
3	95.26
4	94.45
5	96.44
Average	96.462
S.D.	1.7383
% RSD	1.8021

System precision

Six replicate injections of the standard solution at the working concentration showed % RSD (Relative standard deviation) less than 2 concerning peak area for the drug, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in table 2.

Method precision: Method precision was determined by performing assay of the sample under the tests of repeatability (Intra- day precision) at working concentration.

Repeatability: (Intra-day precision) Six consecutive injections of the sample from the same homogeneous mixture at working concentration showed % RSD less than 2 concerning % assay for the drug which indicate that the method developed is precise by the test of repeatability and hence can be concluded that the method will give consistently reproducible results (Table 3).

Linearity

Standard solutions of artemether at different concentrations level were prepared. Calibration curve was constructed by plotting the concentration level of drug versus the corresponding peak area. The results show an excellent correlation between peak area and concentration level of drug within the concentration range (20-200 µg/ml) for the drug and the results as shown in Figure 5. The correlation coefficient of artemether is 0.994, which meet the method validation acceptance criteria and hence the method is said to be linear.

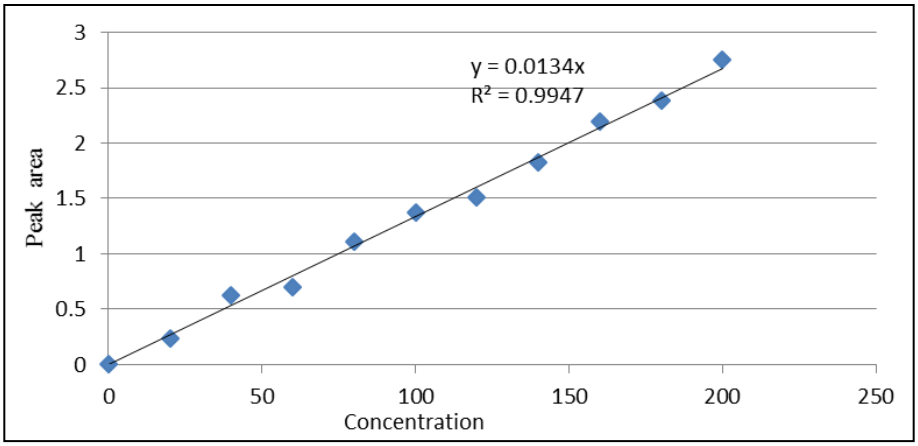

Fig 5: Calibration curve of artemether in plasma

Accuracy

Accuracy was determined by means of recovery experiments by the determination of % mean recovery of sample at three different levels (80%-120%). At each level three determinations were performed. Percent mean recoveries were within limits and the accepted limits of recovery were 98% - 99% (Table 6). All observed data were within the required range which indicates good recovery values and hence the accuracy of the method developed can be confirmed.

Table 6: Results of accuracy studies for artemether

Conc. (µg/ml)	Spiked drug (µg/ml)	Recovered drug (µg/ml)	% Recovery	% RSD
80%	100	98.36	98.36	0.1865
100%	120	119.25	99.63	0.245
120%	140	139.65	99.96	0.178

Sensitivity

The sensitivity of measurement of artemether by use of the proposed method was estimated in terms of limit of quantification (LOQ) and the limit of detection (LOD). The limit of detection (LOD) and limit of quantitiation (LOQ) for artemether was found to be 7.202 µg/ml and 21.831 µg /ml.

All the parameters for validation were found within the required limits.

Conclusion

The artemether was successfully quantified in plasma with good linear relationship. The HPLC-UV method was found to be accurate and precise with a %RSD of less than 2.

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