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Validated HPTLC method of analysis for artemether and its formulations

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Abstract

A simple, sensitive, precise and rapid high-performance thin-layer chromatographic (HPTLC) method of analysis for artemether both as a bulk drug and in pharmaceutical formulations was developed and validated. The method employed TLC aluminum plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene–ethyl acetate–formic acid (8:2:0.3, v/v/v) as mobile phase. Densitometric analysis of artemether was carried out in the reflectance mode at 565 nm. The system was found to give compact spots for artemether (R_f value of 0.50 ± 0.03). The linear regression analysis data for the calibration plots showed good linear relationship with $r^2 = 0.9904$ in the concentration range 200–1000 ng per spot. The mean value of correlation coefficient, slope and intercept were 0.9904 ± 0.011, 7.27 ± 0.11 and 166.24 ± 56.92, respectively. The method was validated for precision, accuracy, recovery and robustness. The limits of detection and quantitation were 65.91 and 197.74 ng per spot, respectively. The method has been successfully applied in the analysis of lipid based parenteral formulations and marketed oral solid dosage formulation.
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Keywords: Artemether; HPTLC-densitometry; Liposomes; Microemulsion

1. Introduction

Artemether ((3*R*, 5*aS*, 6*R*, 8*aS*, 9*R*, 10*S*, 12*R*, 12*aR*)-Decahydro-10-methoxy-3, 6, 9-trimethyl-3, 12-epoxy-12*H*-pyrano [4,3-j]-1, 2-benzodioxepin) (Fig. 1) is a semisynthetic polyoxygenated amorphene containing a peroxide bridge that confers potent antimalarial activity [1]. It is the *O*-methyl ether prodrug of dihydroartemisinin and a derivative of artemisinin (qinghaosu), the principal antimalarial constituent of the Chinese herb *Artemisia annua* (qing hao) [2]. Artemether is active against the erythrocytic stage of multidrug-resistant strains of *Plasmodium falciparum*.

The antimalarial activity has been attributed to chemical activation of the drug within the food vacuole of the intraerythrocytic stage of the parasite; it is proposed that reductive cleavage of the peroxide bridge by heme liberated during digestion of hemoglobin generates free radicals, which induce oxidative stress and alkylate heme and vital parasite proteins [3]. An interaction with membrane phospholipids has also been suggested [4]. The peroxide group in these compounds appears essential for activity, and the peroxide group is retained in the active metabolite, dihydroartemisinin [5].

Because of the promising activity exhibited by artemether against multidrug-resistant strains of *P. falciparum*, several researchers have focused on the development of various analytical methods to determine artemether in different matrices, such as plant extracts, serum, pharmaceutical formulations. These methods include gas chromatography–mass spectrometry (GC–MS) [6], high-performance liquid chromatography (HPLC) based on UV absorption [7–9], chemiluminescence and electrochemical detection [10], high-performance thin-layer chromatography (HPTLC) [11,12] and the capillary electrophoresis techniques [13]. Recently, Gabriëls and Plaizier-Vercammen have reported determination of artemether by using normal phase thin-layer chromatography (NPTLC) [11] using pure chloroform as the mobile phase and also the use of reverse phase thin-layer chromatography (RPTLC) [12] using acetonitrile–water as mobile phase.

Nowadays, HPTLC has become a routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness [14]. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quan-

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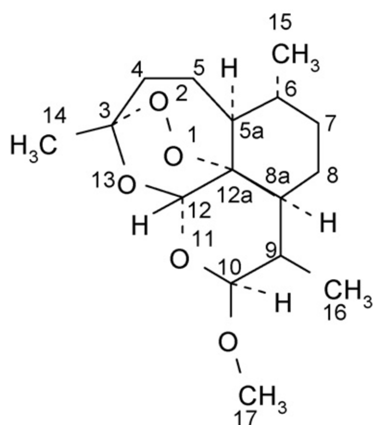

Fig. 1. Chemical structure of artemether.

tity of mobile phase unlike HPLC. This reduces the time and cost of analysis and possibilities of pollution of the environment. HPTLC also facilitates repeated detection (scanning) of the chromatogram with same or different parameters. Simultaneous assay of several components in a multicomponent formulation is possible. The aim of the present work is to develop and validate [15] an accurate, specific and reproducible HPTLC method for determination of artemether as bulk drug and in lipid based parenteral formulations like liposome and microemulsion and also in marketed oral solid dosage formulation (Larither® capsules).

2. Experimental

2.1. Drug and chemicals

Artemether was obtained as kind gift sample from IPCA laboratories, Mumbai, India, and used without further purification, certified to contain 99.98% (w/w). Larither® capsules were procured from market (manufactured by IPCA Laboratories Ltd.). Analytical grade methanol, chloroform, anisaldehyde, sulphuric acid (95–97%, v/v), toluene, formic acid and ethyl acetate, hexane (65–70 °C), acetone and glacial acetic acid were all obtained from Qualigens Fine Chemicals, Mumbai, India.

2.2. HPTLC instrumentation and chromatographic condition

2.2.1. Preparation of visualizing agent

The visualizing agent was prepared by adding 1 ml concentrated sulphuric acid to a solution of 1 ml anisaldehyde in 10 ml methanol. The reagent was freshly prepared before use [16].

2.2.2. HPTLC instrumentation

The chromatographic estimation was performed by spotting standards and extracted samples of artemether on precoated silica gel aluminum plate 60F-254 (10 cm × 10 cm with 250 μm thickness, E. Merck, Darmstadt, Germany, supplied by Anchrom Technologies, Mumbai, India) using a Camag Linomat IV sample applicator (Camag, Muttenz, Switzerland) and a 100 μl Hamilton syringe. The samples, in the form of bands of length 6 mm, were spotted 15 mm from the bottom, 15 mm from left margin of the plate and 10 mm apart, at a constant application rate of 150 nl/s using nitrogen aspirator. Plates were developed using a mobile phase consisting of toluene–ethyl acetate–formic acid (8:2:0.3, v/v/v). Linear ascending development was carried out in 10 cm × 10 cm twin-trough glass chamber (Camag Muttenz, Switzerland) equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature. The length of chromatogram run was 7 cm. Approximately, 10 ml of the mobile phase (5 ml in trough containing the plate and 5 ml in the other trough) was used for each development, which required 8 min. It results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer and treated with anisaldehyde–sulphuric acid reagent for 4 s and heated for 12 min at 110 °C. The slit dimension settings of length 5.00 mm and width 0.45 mm, and a scanning rate of 20 mm/s was employed. The monochromator bandwidth was set at 20 nm.

Densitometric scanning was performed on Camag TLC scanner III in the reflectance mode at 565 nm [13] and operated by winCATS Planar chromatography version 1.1.3.0. The source of radiation utilized was halogen tungsten lamp. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

2.2.3. Calibration curves of artemether

Calibration solutions of artemether in methanol containing concentrations of artemether from 40 to 200 μg/ml were prepared by individual weighing. Five microlitres from each solution was spotted on the TLC plate to obtain final concentration range of 200–1000 ng/spot. Each concentration was spotted two times on the TLC plate. The data of peak area versus drug concentration were treated by linear least-square regression analysis.

2.3. Method validation

The HPTLC method developed was validated for following parameters.

2.3.1. Sensitivity

The sensitivity of the method was determined with respect to LOD, LOQ, linearity range and correlation coefficient. Solutions containing 200–1000 ng of artemether were spotted on TLC plate. The LOD was calculated as 3 times the noise level and LOQ was calculated as 10 times the noise level.

2.3.2. Selectivity

The selectivity of the assay was determined in relation to interferences from formulation ingredients like from liposomes and microemulsions.

2.3.3. Recovery study

Recovery of artemether was determined by spiking artemether in drug free liposomes to obtain three different concentrations covering the low, medium and higher ranges of the calibration curve. The samples were then extracted and analyzed as described in Section 2.2.2. The recovery was calculated by comparing the resultant peak areas with those obtained from pure standards in methanol at the same concentrations.

2.3.4. Precision and accuracy

Different amount of artemether covering low, medium and higher ranges of the calibration curve were spotted on the TLC plate. These spots were analyzed by using above described HPTLC method. Precision was expressed as the percent relative standard deviation (% R.S.D.) and accuracy was expressed as a percentage (observed concentration × 100/theoretical concentration).

2.3.5. Reproducibility

The repeatability was evaluated by analyzing the amount of artemether spotted on TLC plate covering low, medium and higher ranges of calibration curve in replicates ($n = 5$). The intermediate precision was evaluated by analyzing the same amount of analyte over period of 3 days ($n = 7$) and expressed in terms of % R.S.D.

2.4. Analysis of marketed formulation

The developed method can be applied in determination of artemether in Larither® capsules, which is marketed oral solid dosage formulation.

To determine the contents of artemether in capsules (Larither®, Batch No. UY 5007P, Mfg. Date November 2005, Exp. Date October 2007; label claim: 40 mg per capsule), the contents of capsules were emptied and weighed. The drug from the powder was extracted with 10 ml methanol. To ensure complete extraction of the drug, it was sonicated for 30 min. The resulting solution was allowed to settle for about an hour and the supernatant was suitably diluted to give desired concentration (400 ng/10 μl). Ten microlitres of the solution was applied on TLC plate followed by development, visualization with anisaldehyde–sulphuric acid reagent and scanned as described in Section 2.2.2. The analysis was repeated in triplicate. The possibility of excipient interference in the analysis was studied.

3. Results and discussion

The reported methods of artemether estimation like HPLC requires derivatization [8,9] or working at lower wavelength like at 215 nm [7] for sample detection due to lack of UV absorbing chromophore. HPTLC offers several advantages over reported methods. It facilitates automatic application and scanning in situ.

The composition of the mobile phase for development of chromatographic method was optimized by testing different solvent mixtures of varying polarity. Various mobile phases were evaluated (Table 1). Use of chloroform as single component and short saturation time of 15 min give necklace effect. So chloroform–methanol (95:5, v/v), hexane–ethyl acetate (6:4, 7:3, v/v), hexane–acetone (8:2, 9:1, v/v) were tried. The best results were obtained using toluene–ethyl acetate–formic acid (8:2:0.3, v/v/v). This mobile phase showed good resolution of artemether peak from other formulation components or excipients tested as seen in Fig. 2.

Table 1
R_f values of artemether in different mobile phases

S. no.	Composition (v/v) components	Proportions	R_f
1	Chloroform	100	0.53
2	Chloroform–methanol	9.5:0.5	0.76
3	Hexane–ethyl acetate	6:4	0.84
4	Hexane–ethyl acetate	7:3	0.78
5	Hexane–acetone	8:2	0.77
6	Hexane–acetone	9:1	0.79
7	Toluene–ethyl acetate–formic acid	8:2:0.3	0.50
8	Toluene–ethyl acetate–acetic acid	8:2:0.3	0.55

Densitometric scanning of all the tracks showed compound with R_f value 0.50 ± 0.03 (single violet spot), identified as artemether. The present method uses toluene–ethyl acetate–formic acid (8:2:0.3, v/v/v) as the mobile phase for development. The present method is quicker as the time needed for development of plate is reduced considerably to less than half an hour for chamber saturation and development of plate as compared to the previously reported method, which uses pure chloroform and recommends overnight chamber saturation. In the anisaldehyde–sulphuric acid visualizing agent,

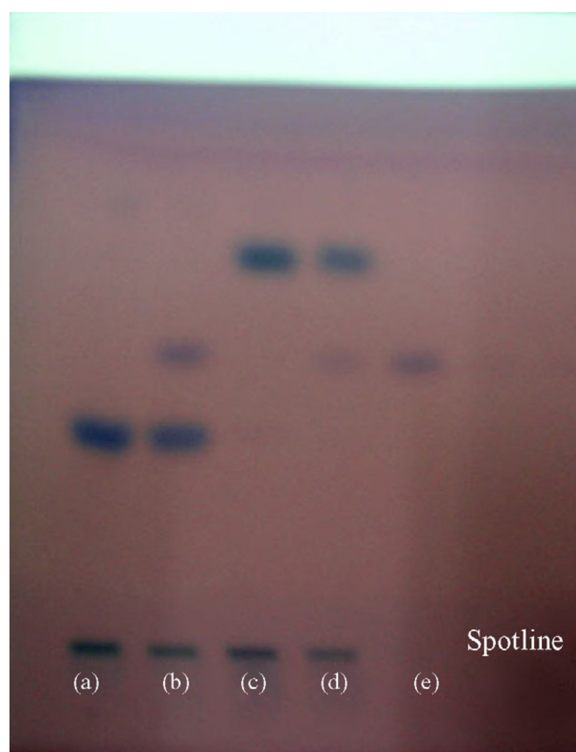

Fig. 2. Photodocumentation of (a) empty liposomes, R_f: 0.36, (b) drug loaded liposomes, (c) empty microemulsion; R_f: 0.66, (d) drug loaded microemulsion and (e) plain drug; R_f: 0.50 using 60F-254 TLC plates, mobile phase toluene–ethyl acetate–formic acid (8:2:0.3, v/v/v).

Table 2
Linear regression data for the calibration curves

Linearity range (ng spot^{-1})	r^2 ± S.D.	Slope	Intercept
200–1000	0.9904 ± 0.0115	7.2696 ± 0.1138	166.243 ± 56.9212

Table 3
R_f values of artemether, liposome component and microemulsion component tested by HPTLC

Compound	R_f
Artemether	0.50
Liposome component	0.36
Microemulsion component	0.66

acetic acid and water were omitted from the composition, which facilitated quick and uniform drying. The method was successfully used in the analysis of artemether from the parenteral dosage forms, liposomes and microemulsions and in case of Larither® capsules without interference of the formulation excipients.

3.1. Sensitivity

Under the experimental conditions employed, the lowest amount of drug which could be detected was found to be 65.91 ng/spot and the lowest amount of drug which could be quantified was found to be 197.74 ng/spot, with relative standard deviation <6%. The calibration curve was found to be linear in the range of 200–1000 ng (n = 3). Peak area and concentration was subjected to least-square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data as shown in Table 2 shows a good linear relationship over the concentration range studied.

3.2. Selectivity

Fig. 3 shows 3-D overlay of HPTLC densitograms of (a) empty liposomes, (b) drug loaded liposomes, (c) empty microemulsion, (d) drug loaded microemlsion and (e) plain drug. The samples were processed as described earlier and 10 μl was spotted on plate and quantified using developed method.

Table 3 shows the R_f values of artemether, liposome components and microemulsion components, which were tested for potential interference with quantification of artemether. The method can also effectively estimate artemether in marketed capsules (Larither®). Thus, at the R_f value of artemether, no interfering peaks are observed thereby confirming the selectivity of the method.

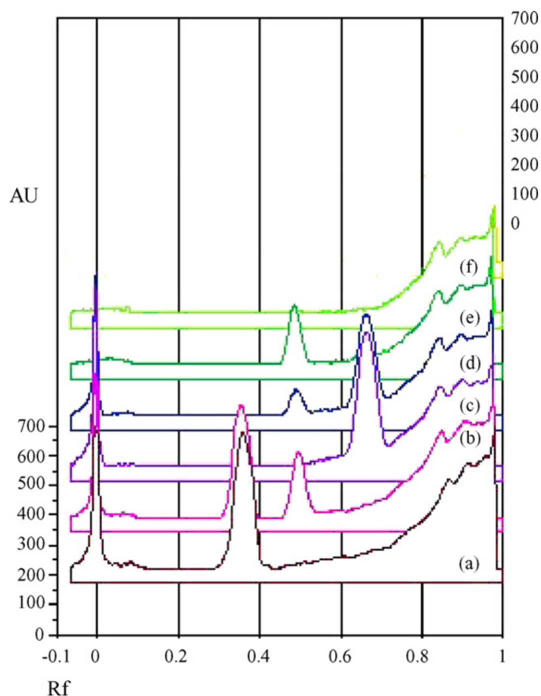

Fig. 3. 3-D overlay of HPTLC densitograms of: (a) empty liposomes, (b) drug loaded liposomes, (c) empty microemulsion, (d) drug loaded microemlsion, (e) plain drug and (f) blank methanol using 60F-254 TLC plates and toluene–ethyl acetate–formic acid (8:2:0.3, v/v/v).

3.3. Recovery study

Results showed high extraction efficiency of artemether from formulation components. The recovery of artemether ranged from 92.21 to 103.12%, average of 97.66%. This confirms that the proposed method can be used for determination of artemether in liposomes formulated in our lab.

Table 4
Precision and accuracy data of TLC method performed on artemether

Parameter	Values		
Actual amount of artemether spotted (ng)	200	600	1000
Amount detected[a] (ng ± S.D.)	189.93 ± 8.86	580.55 ± 25.75	981.42 ± 39.03
R.S.D. (%)	4.67	4.44	3.98
Amount detected[b] (ng ± S.D.)	191.52 ± 6.66	596.60 ± 18.80	1017.38 ± 37.93
R.S.D. (%)	3.48	3.15	3.73

[a] One spot is scanned eight times.
[b] Eight spots scanned once.

Table 5
Accuracy and precision of the assay

Amount of artemether spotted (ng)	Amount detected (ng) (mean ± S.D., $n = 3$)	R.S.D. (%)	Accuracy (%)
200	191.60	0.67	93.76
600	598.57	2.04	99.76
1000	976.65	2.85	97.66

3.4. Precision and accuracy

Five microlitre aliquots of samples containing 200, 600 and 1000 ng artemether were analyzed according to the proposed method. In order to control the scanner parameters, one spot was analyzed several times. By spotting and analyzing the same amount several times ($n = 8$) the precision of the automatic spotting device and the derivatization technique, was evaluated. The relative standard deviation (% R.S.D.) for the analysis of eight replicates indicated good precision for the proposed TLC method (% R.S.D. consistently less than 5) as shown in Table 4. From the results, scanning eight spots in one run is the method of choice.

The result shown in Table 5 depicts good accuracy and high precision. The accuracy was found to be in the range of 93.76–99.76% and % R.S.D. in range of 0.67–2.85.

3.5. Reproducibility

Table 6 shows repeatability and intermediate precision studies of artemether at different levels. The percentage R.S.D. was found to range from 4.15 to 3.18%, averaging to 3.66%.

3.6. Analysis of marketed formulation

The analysis of marketed formulation of artemether capsules (Larither®) showed drug content of 47.60 ± 4.05 mg. The densitogram of the marketed formulation is shown in Fig. 4. The applicability of the method was verified by determination of artemether in pharmaceutical preparations for parenteral use, liposomes and microemulsions developed in our lab. Fig. 3 shows the selectivity of the separation and the specificity of detection. The percent recovery of the proposed method ranges from 92.21 to 103.12% averaging to 97.66%.

Table 6
Precision data of HPTLC assay for artemether

Amount of artemether spotted (ng)	Amount detected (ng) (mean ± S.D.)	R.S.D. (%)
Inter-day ($n = 5$)		
200	192.18 ± 7.79	4.05
600	591.09 ± 21.16	3.58
1000	1011.14 ± 41.96	4.15
Intra-day ($n = 7$)		
200	192.38 ± 7.39	3.84
600	594.16 ± 18.89	3.18
1000	1016.50 ± 36.13	3.55

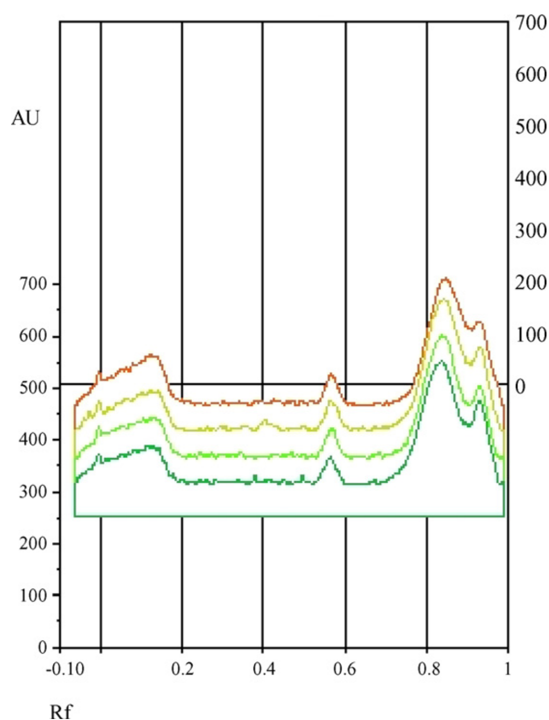

Fig. 4. Densitogram of marketed formulation of artemether, R_f: 0.53 using 60F-254 TLC plates and toluene–ethyl acetate–formic acid (8:2:0.3, v/v/v).

4. Conclusion

The developed HPTLC method combined with densitometry was found suitable for determination of artemether as bulk drug, in lipid based parenteral formulations like liposomes and microemulsion and also in marketed solid dosage formulation (Larither® capsules) without any interference from the excipients. Statistical analysis proves that the method is repeatable and selective for the analysis of artemether. Its advantages are low cost of reagents, speed and simplicity of sample treatment, satisfactory precision and accuracy.

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