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RP-HPLC method development and validation for the estimation of artemisinin in bulk drug

Ashish Sethiya

Bhupal Nobles' University, Udaipur, Rajasthan, India

Corresponding author email: ashish.brncop@meu.edu.in

R. P. S. Rathore

Department of Pharmacy, B. N. University, Udaipur, Rajasthan, India

Abstract---Artemisinin is a sesquiterpene lactone obtained from sweet wormwood, *Artemisia annua*, which is used as an antimalarial for the treatment of multi-drug resistant strains of falciparum malaria. It has a role as an antimalarial and a plant metabolite. Several artemisinin derivatives have been developed for clinical use in prevention and treatment of malaria, some of which have been linked to rare instances of acute liver injury. A reversed-phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the estimation of artemisinin in bulk drug. The separation was achieved on Thermo C₁₈ analytical column (250 mm × 4.6 mm i.d., 5.0µm) using 20mM KH₂PO₄: acetonitrile in the ratio of 15:85v/v (pH adjust with 4.0 with OPA) in the ratio 15:85 v/v as mobile phase and at a flow rate of 1.0 ml/min. Detection was carried out using a UV detector at 303nm. The total chromatographic analysis time per sample was about 15 min with artemisinin eluting at retention time of about 11.254 ± 0.002min. The method was validated for accuracy, precision, specificity, linearity and sensitivity. Validation studies demonstrated that this HPLC method is simple, specific, rapid, reliable and reproducible. The standard curve was linear over the concentration range of 5-25µg/ml with r² close to one (0.999). The limit of detection (LOD) and limit of quantitation (LOQ) obtained for lumefantrine were 0.15µg/ml and 0.45µg/ml respectively. The high recovery and low relative standard deviation confirm the suitability of the proposed method for the determination of artemisinin in bulk drugs.

Keywords---artemisinin, RP-HPLC, ICH guidelines, antimalarial agent.

Introduction

Malaria is a life-threatening parasitic disease that occurs in many tropical and subtropical areas especially the Africa region where about 92% of the global malaria cases were recorded in 2017. The 2018 World Malaria Report estimated that 435,000 deaths occurred globally in the year 2017, of which an estimated 93% were recorded in the World Health Organization (WHO) Africa Region [1]. Malaria case management is guided by the infecting *Plasmodium* species, clinical status of the patient and drug susceptibility of the infecting parasite [2]. Current WHO guidelines for treatment of *falciparum* malaria employ artemisinin-based combination therapy (ACT). ACT is based on the use of two drugs with different modes of action: an artemisinin-derivative that rapidly clears asexual blood stage parasites and gametocytes, as well as a partner drug that has a longer half-life, thus eliminating residual parasites [3]. Artemisinin is the bioactive compound produced by the plant *Artemisia annua* L. Artemisinin has saved the lives of millions of malarial patients worldwide and served as the standard regimen for treating *Plasmodium falciparum* infection [4]. With 500 species, the genus *Artemisia* L. is the largest and the most widely distributed genus of the Asteraceae, and Central Asia is the center of origin and diversification of the genus [5]. Many *Artemisia* species grow in Tajikistan [6]. The genus *Artemisia* is known to possess rich phytochemical diversity [7, 8]. Almost 600 secondary metabolites have been characterized from *A. annua* alone [9] Artemisinin is the shining example of a phytochemical isolated from *A. annua*, and is widely used in the treatment of malaria. Artemisinin is a natural sesquiterpene lactone with an unusual 1,2,4-trioxane substructure (Figure 1). It is soluble in most aprotic solvents and is poorly soluble in water. It decomposes in protic solvents, probably by the opening of the lactone ring [10]. The artemisinin biosynthesis proceeds via the tertiary allylic hydroperoxide, which is derived from the oxidation of dihydroartemisinic acid [9]. The mechanism of artemisinin action is controversial. It is related to the presence of an endoperoxide bridge, which by breaking creates a powerful free radical form of the artemisinin, which attacks the parasite proteins without harming the host [11]. The empirical formula of artemisinin is $C_{15}H_{22}O_5$ with a molecular weight of 282.3 [12]. The bioavailability of artemisinin after oral administration is 32% [13]. The low bioavailability of artemisinin after oral administration is not due to its inability to cross the intestinal membrane [14], rather it has been attributed to its low aqueous solubility. Since dissolution is the rate-limiting step for the bioavailability of artemisinin following oral administration, dissolution rate studies are useful in determining artemisinin bioavailability. Due to its low solubility, artemisinin concentrations in the dissolution medium are very low, therefore necessitating a sensitive method for its analysis. Several methods for the analysis of artemisinin have been published, but standard detection methods are hindered by artemisinin lack of a suitable chromophore for UV detection. These methods include gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), ultraviolet spectroscopy (UV), and immunoassays [15-29]. The most recent publication on the quantitative analysis of artemisinin is by LC-MS with selective ion monitoring (SIM) [30]. This paper describes the development and validation of reliable, simple, robust, time and money saving reversed phase HPLC method,

using PDA detection, for the estimation of artemisinin in bulk drugs. The developed method validated according to ICH guidelines [31].

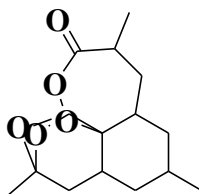


Figure 1 Chemical structure of artemisinin

Materials and Methods

Instrumentation

Liquid chromatographic system from Waters model no 784 comprising of manual injector, water 515 binary pump for constant flow and constant pressure delivery and UV-Visible detector connected to software Data Ace for controlling the instrumentation as well as processing the generated data. Weighing was done on a Digital Micro Balance (CX-265) manufactured by Citizen Scale (I) Pvt. Ltd.

Reagents and chemicals

Analytically pure sample of artemisinin was a generous gift from Salvavidas Pharmaceutical Private Limited, Surat, India along with their analytical reports. Potassium di hydrogen phosphates (AR grade), disodium hydrogen phosphate (AR grade), OPA and acetonitrile (HPLC Grade) was purchased from E. Merck Ltd. Worli, Mumbai, India. All other chemical used were of analytical grade. Triple distilled water was used for whole experiment was generated in house.

Diluents

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect retention and resolution of analyte. After various trials, 0.1 N HCl was used as diluents.

Selection of mobile phase

Initially to estimate artemisinin simultaneously, number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was 20mM KH_2PO_4 : acetonitrile (pH 4.0 with orthophosphoric acid) in the ratio 15:85 v/v run as isocratic system. The mobile phase was filtered through 0.45 m filter paper and then degassed by Sonication. Flow rate employed for analysis was 1 ml/min.

Chromatographic conditions

The isocratic mobile phase consisted of 20mM KH_2PO_4 : acetonitrile (pH 4.0 with orthophosphoric acid) in the ratio 15:85 v/v, flowing through the column at a constant flow rate of 1.0 ml/ min. The mobile phase was filtered through nylon 0.22 μm membrane filters and was degassed before use (30 min). A Thermo (C-18) column (5 μm , 250mm x 4.60mm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity and selectivity of method for drugs, 303.0 nm was selected as the detection wavelength for UV-Visible detector.

Standard preparation

Preparation of stock solution

Accurately weighed 10 mg API of artemisinin was transferred into 10 ml volumetric flask separately and added 5ml of 0.1 N HCl as diluents, sonicated for 20 minutes and volume was made up to 10ml with 0.1 N HCl to get concentration of solution 1000 $\mu\text{g}/\text{ml}$ (Stock-A)

Preparation of sub stock solution

5 ml of solution was taken from stock-A of both the drug and transferred into 50ml volumetric flask separately and diluted up to 50 ml with diluent (0.1 N HCl) to give concentration of 100 $\mu\text{g}/\text{ml}$ of artemisinin respectively (Stock-B).

Preparation of different solution

0.5ml, 1.0ml, 1.5ml, 2.0ml and 2.5ml of stock-B were taken separately in 10 ml volumetric flask and volume was made up to 10ml with (0.1 N HCl). This gives the solutions of 5 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$, 15 $\mu\text{g}/\text{ml}$, 20 $\mu\text{g}/\text{ml}$ and 25 $\mu\text{g}/\text{ml}$, for artemisinin.

Results and Discussion

Chromatography

The mobile phase was chosen after several trials with methanol, isopropyl alcohol, acetonitrile, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting of 20mM KH_2PO_4 : acetonitrile (pH 4.0 with orthophosphoric acid) in the ratio 15:85 v/v was selected to achieve maximum separation and sensitivity. Flow rates between 0.5 and 1.5 min were studied. A flow rate of 1 ml/min gave an optimal signal-to-noise ratio with a reasonable separation time. Using a reversed-phase C_{18} column, the retention times for artemisinin was observed to be $11.254 \pm 0.002\text{min}$. Total time of analysis was less than 15min. The maximum absorption of artemisinin was detected at 303nm and this wavelength was chosen for the analysis. Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present such as impurities, degradation products and matrix components Fig. 2.

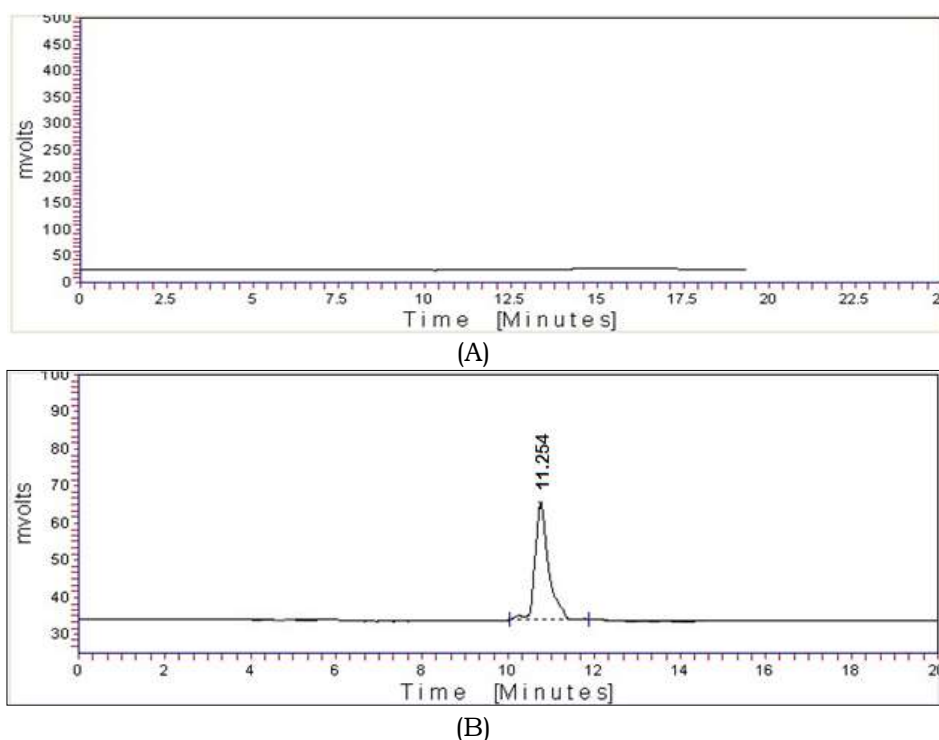


Figure 2 Chromatograms of (A) Blank mobile phase (B) artemisinin (15µg/ml) as reference substances

System suitability

System suitability parameters such as number of theoretical plates, HETP and peak tailing are determined. The results obtained are shown in Table 1. The number of theoretical plates for artemisinin was 2488.00.

Table 1 Results of system suitability parameters

Parameters	Artemisinin
AUC*	273.394
No. of Theoretical Plates	2488.00
Tailing Factor*	1.053
Retention time*	11.254 ± 0.002
Calibration range (µg/ml)	5-25

*Each value is the mean ± SD of six determinations

Linearity

The calibration curve was linear over the concentration range of 5-25µg/ml for artemisinin. The linearity was represented by a linear regression equation as follows:

$$Y (\text{artemisinin}) = 26.74\text{conc} + 0.357 \quad (r^2 = 0.999)$$

Accuracy

Recovery studies were performed to calculate the accuracy of developed method to reanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed. The value of percentage RSD was found less than 2 (0.281, 0.282 and 0.281) show good recovery at all three level 80, 100 and 120% respectively. Each level was made in triplicate Table 2.

Table 2 Results of recovery study

% Level	% Mean \pm SD*
	Artemisinin
80%	99.32 \pm 0.279
100%	99.07 \pm 0.279
120%	99.41 \pm 0.279

* Value of three replicate and three concentrations.

Precision

Repeatability

Five dilutions in three replicates were analyzed in the same day for repeatability and results were found within acceptable limits (RSD < 2) as shown in Table 3.

Intermediate precision

Five dilutions in three replicates were analyzed on two different days and by two analysts for day-to-day and analyst-to-analyst variations and results were found within acceptable limits (RSD < 2) as shown in Table 3.

Robustness

As per ICH norms, small, but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was change from, 20mM KH₂PO₄: acetonitrile (15:85 % v/v), to (20: 80% V/V) and method is found robust as RSD is again found < 2.0 Table 3.

Table 3 Statistical data for precision and robustness

Statistical parameter	Artemisinin		
	Mean*	S.D*	R.S.D*
Repeatability	98.971	0.078	0.079
Intermediate Precision (I) (A day to day)	99.423	0.148	0.148
(II) Analyst to Analyst	99.059	0.112	0.113
Robustness	98.906	0.103	0.104

*Mean of 15 determinations (three replicates at five concentration level)

Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve Table 4.

Table 4 LOD and LOQ

Name	LOD ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)
Artemisinin	0.15	0.45

Conclusion

The proposed HPLC method was validated as per the International Conference on Harmonisation (ICH) Q2B Guidelines, and was found to be applicable for routine quantitative analysis of artemisinin by HPLC in pharmaceutical dosage form. The results of linearity, precision, accuracy and specificity, were proved to be within the limits. The method provides selective quantification of artemisinin with no interference from other formulation excipients. The proposed method was highly reproducible, reliable, rapid, robust and specific. Therefore, a high percentage of recovery allows its application for the routine determination of artemisinin in the pharmaceutical dosage form.

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