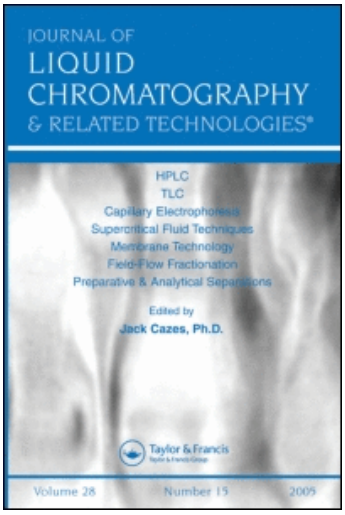

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Maher M. El-domiaty[a]; Ibrahim A. Al-meshal[a]; Farouk S. El-feraly[a]
[a] Department of Pharmacognosy, College of Pharmacy King Saud University Riyadh, Saudi Arabia

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ARTEMISITENE IN ARTEMISININ

MAHER M. EL-DOMIATY*, IBRAHIM A. AL-MESHAL, AND FAROUK S. EL-FERALY
Department of Pharmacognosy
College of Pharmacy
King Saud University
Riyadh 11451, Saudi Arabia

ABSTRACT

A new, simple and selective reversed-phase HPLC assay is developed for the determination of the clinically undesirable artemisitene in the antimalarial agent artemisinin (qinghaosu). It involves the use of an internal standard (santonin) and the determination time is less than 5 minutes. Detection was accomplished using a UV detector set at 216 nm and limits were as low as 15ng for a 10µl injection. Being simple and selective this method is particularly useful for the routine analysis of artemisinin to check its purity. In addition, the method can be used for preparative scale purification of these compounds. It has been applied for the evaluation of crystalline samples of artemisinin without prior preparation.

*Address for correspondence.

INTRODUCTION

Artemisinin (also called artemisinine or arteannuin and is often referred to as qinghaosu) is a sesquiterpene lactone with an unusual endoperoxide group. It is the clinically active antimalarial constituent isolated from the Chinese traditional herb.Qinghao or Artemisia annua L. (Family Compositae) (1).

Several thousand malaria cases in China have been successfully treated with artemisinin, including those caused by both chloroquine-sensitive and chloroquine-resistant strains of Plasmodium falciparum. Interest in the antimalarial potential of artemisinin as well as its derivatives and analogs is currently the subject of numerous investigations (2,3).

In addition to artemisinin, A. annua elaborates the dehydro analog artemisitene (4), albeit in smaller yields. This compound was found to be less active as an antimalarial agent than artemisinin (5). In addition, the presence of an α,β-unsaturated carbonyl in this compound might confer upon it certain undesirable biological activities such as allergenicity or mutagenicity (6). Even after repeated crystallization of artemisinin, artemisitene co-crystallizes with it and sharp melting samples of artemisinin were claimed to contain as much as 10% of artemisitene (4).

Several HPLC methods have been employed for the determination of artemisinin and its derivatives in

biological fluids (7,8) or in plant extracts (4, 9-11) The only analytical technique available for the determination of artemisitene involved an electrochemical detection HPLC (4). This detection required tedious and complicated procedures to exclude oxygen and maintain the oxygen free state of both the buffered mobile phase and the sample to be injected. The many precautions (4) required throughout the analysis, in addition to the unavailability of such a detection system in many laboratories, represented additional difficulties for routine analysis of artemisinin using this method. Therefore, it was necessary to develop a method which is simple, selective, rapid and sensitive to monitor the concentrations of artemisitene in artemisinin, before the later is used clinically or used for preparation of the more potant semisynthetic derivative arteether which is currently being evaluated by World Health Organization as antimalarial drug (3). This manuscript describes such a method, which has also been adapted to be an easy and rapid method for the HPLC separation and purification of artemisinin and artemisitene on a preprative scale.

MATERIAL AND METHODS

Instruments

The HPLC system consisted of a Waters Modular System (Waters Asocc. Inc., Milford, MA, U.S.A.), fitted

with a model M-45 solvent delivery system attached to an automated controller model 680; a model U6K injector with a 2 ml sample loop; a Lamoda-Max model 481 LC spectrophotometer and a data module model 730. Chromatography was carried out on a stainless steel column (30 cm X 3.9 mm i.d.) packed with reversed-phase μBondapak C_{18} (particle size 10μm)

Chemicals

HPLC-grade acetonitrile and doubly distilled water were used. All solvents were degased and filtered through Durapore filters 0.22μX 47 mm using a Waters solvent clarification kit.

Crystalline artemisinin (containing artemisitene as an impurity) was isolated from either locally grown A. annua (12) using a literature procedure (13) or from plant material grown in University, Mississippi, U.S.A. and harvested at the preflowering stages in August 1984. It was purified by preparative HPLC on a μBondapack C_{18} column (7.8mm X 30cm) using 15% aqueous acetonitrile as an eluent at a flow rate of 5ml/min. (R_t of artemisinin and artemisitene were 7.3 and 6.2 min., respectively). This treatment provided also pure artemisitene, which was also obtained by chemical synthesis (12).

Santonin was of the extra pure grade (Merck, Germany). All reference compounds were checked for

homogeneity and purity by determining their physical constants and by spectral and HPLC analysis.

HPLC Operating Conditions

The mobile phase consisted of a mixture of acetonitrile and water (65:35), at a flow rate of 1.5 ml/min. Wavelength of detection was 216 nm and 0.01 (AUFS) for sensitivity. All chromatographic analyses were performed isocratically and at ambient temperature.

Preparation of Internal Standard Solution

A stock solution was prepared by dissolving an accurately weighed quantity (100 mg) of the internal standard, santonin, in 100 ml of acetonitrile. This stock solution was diluted to give a working concentration of 0.15 mg/ml of santonin in acetoni-trile.

Preparation of Standard Solutions of Artemisitene

A stock solution of artemisitene (1 mg/ml) was prepared by dissolving an accurately weighed amount (100 mg) of artemisitene in 100 ml of acetonitrile. This solution was diluted to a concentration of 0.1 mg/ml with acetonitrile and used for the preparation of the standard curve as described below.

Calibration Graphs of Artemisitene

Six aliquots equivalent to 25, 50, 100, 150, 200 and 250 µg of artemisitene (using the previously prepared stock solution) were transfered to 10-ml volumetric flasks. A 1.5 ml of the stock solution of the internal standard (0.15 mg/ml) was then added. The solutions were then brought to volume with acetonitrile and thoroughly mixed to obtain standard solutions containing 2.5, 5.0, 10, 15, 20 and 25 µg/ml of artemisitene and 22.5 µg/ml of internal standard. A volume of 10 µl of each solution was injected in triplicates onto the column to obtain a calibration graph.

Construction of The Calibration Graph

Three injections of each of the six standard solutions containing artemisitene and santonin were used to establish linearity and response ratios. The calibration curve (Fig. 2) was constructed by plotting the ratio of peak area of artemisitene to that of internal standard versus the concentration of artemisitene.

Samples Preparation

Two crystalline samples of artemisinin of different sources were separately dissolved in acetonitrile to give a concentration of 10 mg/ml. A

volume of 1 ml of this solution was transferred to a 10 ml volumetric flask and 1.5 ml of the stock solution of the internal standard were added and the volume was brought to 10 ml using acetonitrile. An appropriate volume (usually 10 µl) of this solution was injected into the HPLC system.

RESULTS AND DISCUSSION

The chromatographic separation of artemisinin and artemisitene was carried out under a variety of conditions. The selection of a suitable eluent was studied by investigating combinations of organic solvents and water.

Initial work was also directed towards the separation of artemisitene, artemisinin and the internal standard. A chromatographic system consists of µBondapack C_{18} reversed-phase column and a mobile phase of acetonitrile - water (65:35) provided the best separation, as shown in Fig. 1A. The retention times (R_t) of artemisitene, artemisinin and the internal standard, santonin, were 4.30, 4.90 and 3.02 minutes, respectively. The separation was sufficiently good and reproducible to permit quantitative work. The capacity factors (K') of artemisinin, artemisitene and internal standard were 2.8, 2.4 and 1.4, respectively, which are in the optimum range for quantitative analyses (14).

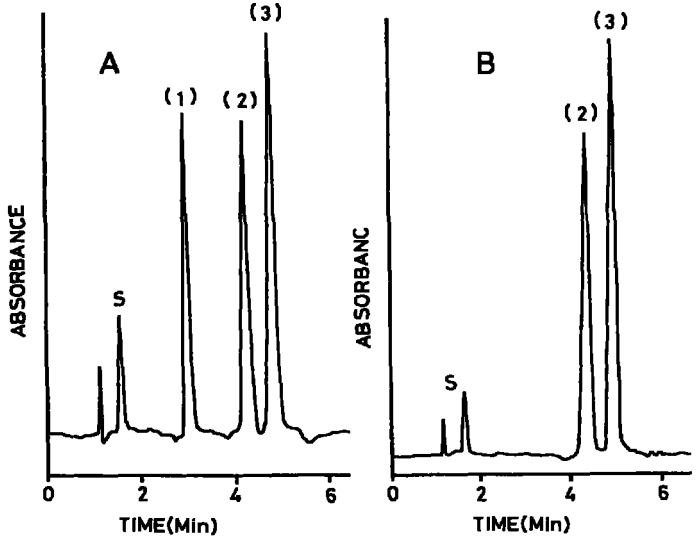

Fig. 1. (A) HPLC separation of pure reference samples of artemisitene (2), artemisinin (3) and the internal standard, santonin (1). (B) A chromatogram of a crystalline sample of artemisinin (3) showing the presence of artemisitene (2). Chromatographic conditions: Column, μBondapack C18 (30cm × 3.9 mm i.d.); mobile phase, acetonitrile:water (65:35) at 1.5 ml/min. flow rate; detection, UV at 216 nm. S - solvent peaks.

Santonin fulfilled the requirement for a good internal standard (14), as it is structurally similar to both artemisinin and artemisitene, eluted close to both of them (K' values were close) with a well resolved peak and is commercially available in a highly pure form.

As the absorption maximum of artemisinin and artemisitene occurs at about 210-220 (7), the UV absorption of the chromatographic solvents becomes a determining factor for the sensitivity of detection (14). However, the transparency of the solvents is greater in the employed reversed-phase system, which permitted detection at 216 nm, with ϵ values of 140 and 5900 for artemisinin and artemisitene, respectively. This resulted in a linear response with an approximate detection limit of 15 ng of artemisitene (per 10 µl injection at a detector sensitivity of 0.01 AUFS). Higher sensitivity may be obtained by the use of larger injection volumes.

The calibraton graph (Fig. 2) was constructed by plotting the ratio of peak area of artemisitene to that of santonin (internal standard) against the amount of the former where a good linear response was observed in the range of 25 to 250 ng of artemisitene, with an excellent correllation coefficient ($r=0.999$). This calibration curve was used for the determination of ar-

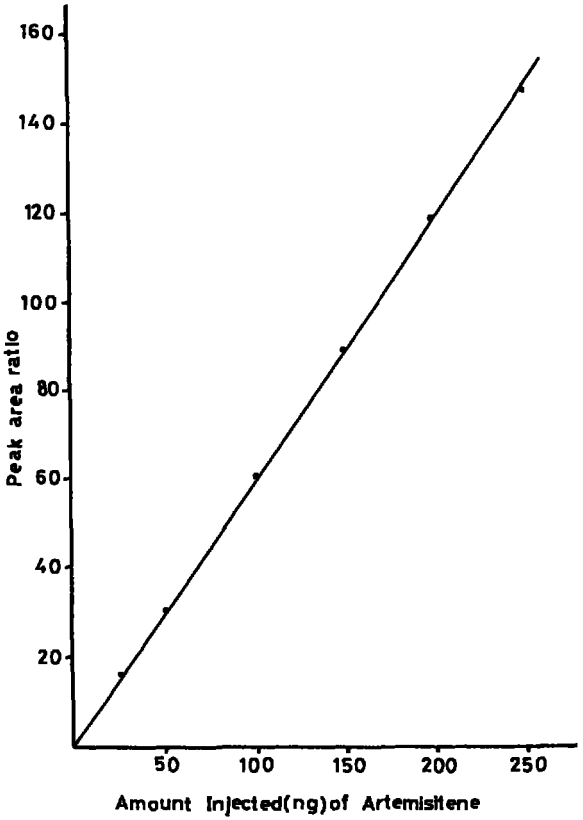

Fig. 2 Calibration curve : Peak area ratio (artemisitene/internal standard) Vs injected amount of artemisitene (ng) - Chromatographic conditions : refer to Fig. 1.

temisitene in the two crystalline samples of artemisinin of different geographical origins, by simply dissolving the samples in acetonitrile and direct injection into the HPLC system. One sample was isolated from A. annua grown locally (Riyadh, Saudi Arabia) (12), and the other one was isolated from a variant grown in University, Mississippi, U.S.A. An HPLC chromatogram of the local crystalline sample of artemisinin is shown in Figure 1B, the presence of artemisitene was evident. The second crystalline sample (from USA) showed a similar chromatogram and hence was not included in the figure.

It is worthwhile, to point out that the presence of artemisitene in artemisinin is not desirable as it is less potent than artemisinin (3,5).

The results showed that, while the American sample had 1.19% of artemisitene, the content of the local sample was a little higher at 1.99%. These results were based on the average of eight separate determinations for each run with a relative standard deviation of 0.01% and 0.08% for the American and the local samples, respectively.

The method was also exploited, with a slight modification, for the separation and purification of both artemisinin and artemisitene. A semi-preparative HPLC column and 15% water in acetonitrile as a mobile

phase under isocratic mode were used as described in "Experimental". The previously reported (4) method for this purpose utilized a gradient elution system, a technique that is not optimum (14) for preprative work. This report provides a much simpler and convenient preparative HPLC method for artemisinin and artemisitene in a short elution time (less than 8 min.).

CONCLUSION

This study has provided a very simple, quick, selective and sensitive HPLC method for the qualitative and quantitative evaluation of the antimalarial drug artemisinin concerning the presence of the undesirable impurity artemisitene. The method provides a good tool for a routine determination of artemisitene in artemisinin, in less than 5 minutes, with a great degree of precision. The method may also be extended for monitoring the artemisitene contents of plant extracts as there was no interferences of other related compounds present in A. annua (13) including the major sesquiterpene lactones artemisinic acid and arteannuin B.

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