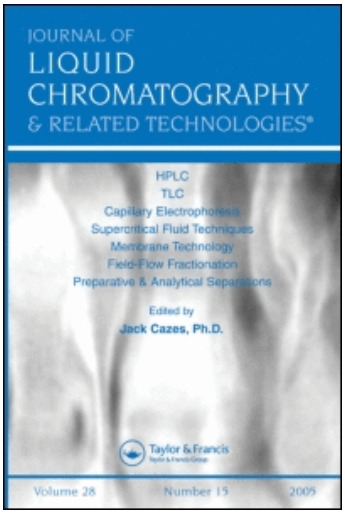

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Determination of Artemisinin in *Artemisia annua L.* by Reversed Phase HPLC

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Abstract: Artemisinin is a compound of current interest in the treatment of drug-resistant malaria. An accurate, sensitive, and reproducible reversed-phase HPLC with UV detection method is described for the determination of artemisinin in the herb of *Artemisa annua L.* Artemisinin in the extracts was converted into a UV-absorbing compound, Q260 by being treated with 0.2% (by weight) NaOH solution and then 0.08 M acetic acid. A reversed-phase C_{18} silica column (Discovery 250 × 4.6 mm, 5 μm) was selected as a fixed phase, and kept at 30°C. The mobile phase was 45/10/45 (by volume) methanol/acetonitrile/0.9' mM Na_2HPO_4-3.6 mM NaH_2PO_4 buffer (pH 7.76). The flow rate was 0.5 mL/min. The detection wavelength was 260 nm. The standard calibration curve was linear with correlation coefficient 0.9996. The artemisinin content in the herb of *Artemisa annua L.* was 0.652%. The relative standard deviation and recovery were 1.1% and 98.9%, respectively.

Keywords: Artemisinin, HPLC, UV detection

INTRODUCTION

Artemisinin, also called qinghaosu (QHS), is a naturally occurring sesquiterpene lactone endoperoxide isolated from *Artemisa annua L.* and chemically characterized by Chinese scientists during the 1970's.[1] It has an empirical formula of $C_{15}H_{22}O_{15}$ and possesses a peroxide bridge (C-O-O-C) to which its antimalarial properties are attributed (see Fig. 1). Artemisinin is a new antimalarial drug that has been shown to be effective against the erythrocytic

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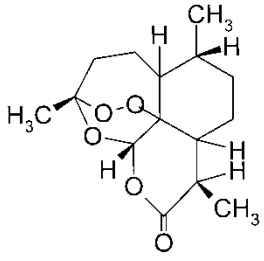

Figure 1. Structure of artemisinin (qinghaosu, QHS).

stages of the plasmodial parasite, even against strains that have developed resistance to other currently available drugs such as chloroquine. This is particularly important since malaria is still a major health problem in many areas of the world.[2]

Analysis of artemisinin is a challenging problem as the compound is unstable, the content in the plants is low, the intact molecule has poor staining characteristics, and other constituents in the plant interfere with the detection. Artemisinin has been detected and quantified by different methods, e.g., thin layer chromatography (TLC), gas chromatography (GC),[3] GC combined with mass spectrometry (GC-MS),[4] tandem mass spectrometry (MS-MS),[5] HPLC with UV detection (HPLC-UV),[6,7] and with electrochemical detection (HPLC-EC).[8]

For the methods developed so far, HPLC with UV detection was the most suitable method to determine artemisinin content in crude plant extracts. Zeng and co-workers[6] reported that treating artemisinin with sodium hydroxide solution gave a resultant, called Q292, having a maximum absorbance at 292 nm. Q292 could be further converted into another compound, called Q260, having a maximum absorbance at 260 nm, by acidifying its solution (see Fig. 2). Therefore, Q260 could be used to determine the artemisinin content.

Zhao and Zeng[7] used 55/45 (by volume) methanol/0.01 M phosphate buffer as the mobile phase to determine artemisinin content in crude plant extracts. However, the solubility of some constituents in the plant in the mobile phase was so small that the baseline appeared to drift during analysis.

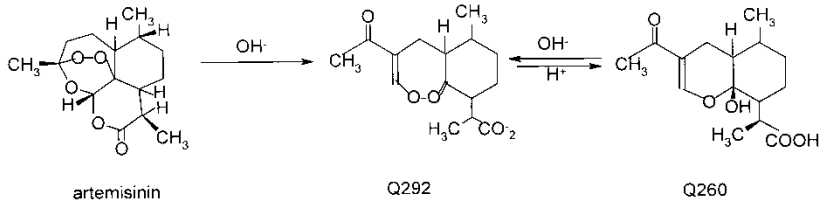

Figure 2. Schematic diagram of artemisinin conversion to Q260.

Thus, the method is not suitable to routine analysis because of its instability. Furthermore, the high concentration of phosphate solution was harmful to the HPLC pump. The purpose of the present investigation is to establish an accurate, sensitive, and stable analytical method for artemisinin in plants.

EXPERIMENTAL

HPLC System

The Waters HPLC system was equipped with a 515 programmable pump, 717plus autosampler, and 996 photodiode array detector. A Discovery 250 × 4.6 mm, reversed-phase C_{18} silica column (5 μm, Supelco, Shanghai, China) was used. The chromatographic system was controlled by using the Millennium chromatography manager software (version 3.20).

Chemicals

The herb, *Artemisia annua L.*, was kindly provided by Holley (Jishou) Pharmaceutical Limited Company (Hunan, China). Standard artemisinin was purchased from Sigma Corporation with purity of 98%. Methanol, ethanol, and acetonitrile were chromatographic grade. Ultrapure water was used. All other chemicals were analytical reagent grade.

Preparation of Standard Solutions of Artemisinin

Standard solutions of artemisinin were prepared by dissolving an accurately weighed quantity (about 100 mg) of artemisinin in ethanol, and adjusted to the 100 mL mark. Five aliquots equivalent to 0, 0.50, 1.00, 2.00, and 5.00 mL of the above solution were pipetted into five 50 mL flasks, respectively. The solutions were then diluted to 5.0 mL with ethanol, using a pipette, and mixed with 20 mL of 0.2% (by weight) NaOH solution, respectively. These mixtures were then warmed in a water bath at 45°C for 30 min to obtain Q292. After being cooled to room temperature with water, these mixtures were neutralized and adjusted to mark with 0.08 M acetic acid to obtain the resulting compound Q260. These solutions were analyzed directly by HPLC.

Extraction Procedure and Sample Preparation

The herb of *Artemisia annua L.* was dried naturally and ground to about 0.25 mm. This powder of 5.000 g was extracted in a Soxhlet extractor with

petroleum ether (30–60°C) 200 mL for 6 h. The extraction solution was vacuum distilled in a rotatory evaporator. After it was cooled to room temperature, the residue was dissolved in ethanol and vacuum filtered. The filtrate was concentrated and transferred into a 50 mL flask. The solution was then adjusted to mark with ethanol, pipetted 10.00 mL above solution into 50 mL flask, then reacted with 0.2% (by weight) NaOH. Finally, it was acidified and adjusted to mark with 0.08 M acetic acid as described before. The sample was a Q260 solution and taken directly for chromatographic analysis.

HPLC Operation Conditions

The mobile phase was 45/10/45 (by volume) methanol/acetonitrile/0.9 mM Na_2HPO_4-3.6 mM NaH_2PO_4 buffer (pH 7.76), with a flow rate of 0.5 mL/min. It was filtered and degassed before use. Detection wavelength was 260 nm. Each sample was filtered by a 0.45 μm filter, and injected 10 μL each time. All chromatographic analysis were performed isocratically and at 30°C.

RESULTS AND DISCUSSION

Chromatogram

At HPLC operation conditions described above, the retention time was 6.30 min for intact QHS (a) (see Fig. 3A). The consistency of chromatograms of Q260 (b) and blank solution(c) around 6.30 min suggested that the conversion of artemisinin to Q260 was complete. The completeness of conversion assured the possible validity of the analysis. The peak at 12.5 minute in Fig. 3B was an unknown impurity in the standard artemisinin.

Figure 4 is chromatograms of sample solutions. The peak at 16.85 min was identified as a single Q260, not overlapped with other compounds. Impurities were eluted much earlier than Q260. This assured the possible validity of the analysis as well. Moreover, the retention time of Q260 and the baseline were extraordinarily stable although the analysis was repeated many times. This suggested that the method was reproducible and suitable to routine analysis.

The UV absorption spectra of Q260 and intact artemisinin are shown in Fig. 5. The absorption coefficients of Q260 and artemisinin are 1.27×10^4 L/(mol · cm) and 3.7×10^2 L/(mol · cm), respectively.

Calibration Curve

Five standard solutions were prepared, at concentrations between 0 and 104.66 μg/mL. The calibration curve was obtained by plotting the

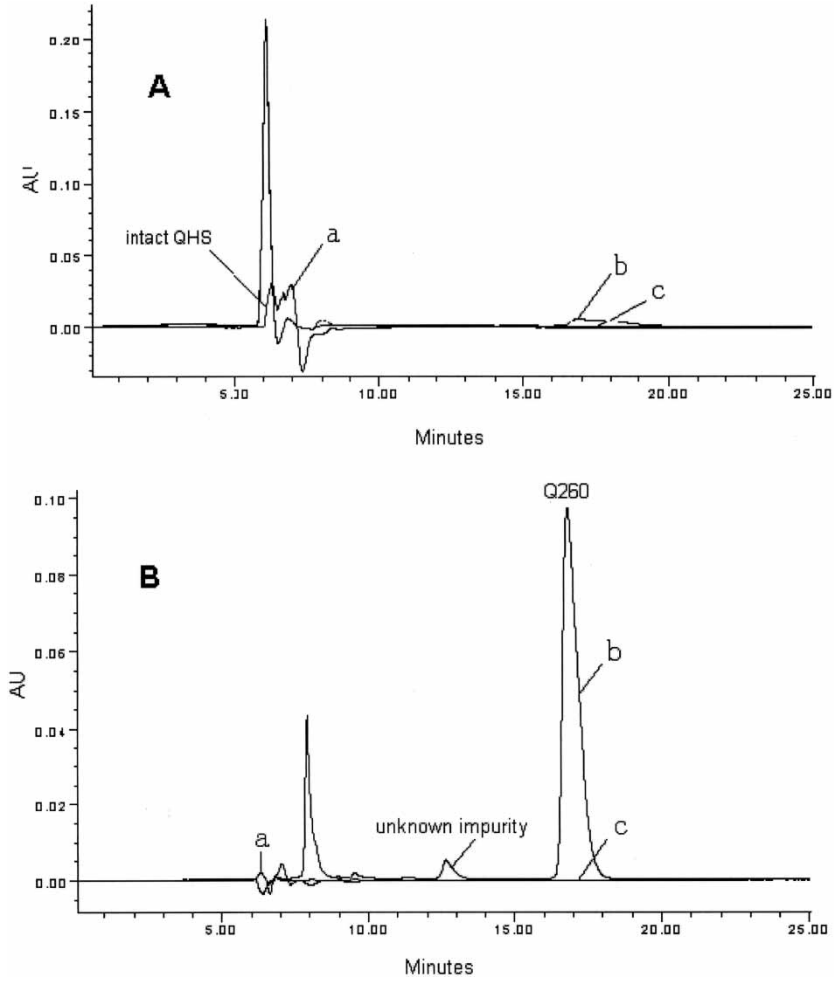

Figure 3. Chromatogram of artemisinin. A: scanning with 203 nm, B: scanning with 260 nm. a: intact QHS (in ethanol), b: Q260 (standard solution, 104.66 μg/mL), c: blank solution. Column: C_{18} (250 mm × 4.6 mm i.d.). Mobile phase: 45/10/45 (by volume) methanol/acetonitrile/0.9 mM Na_2HPO_4-3.6 mM NaH_2PO_4 buffer (pH 7.76). Flow rate: 0.5 mL/min. 10 μL injected.

chromatography peak area against the concentration (see Fig. 6) where a good linear response was observed. Linear regression analysis resulted in the equation with an excellent correlation coefficient ($R = 0.9996$): $A = 3.43 \times 10^4 C$, where A and C were peak area (AU · s) and concentration (μg/mL), respectively. The calibration curve and linear equation were used for the determination of artemisinin in the samples.

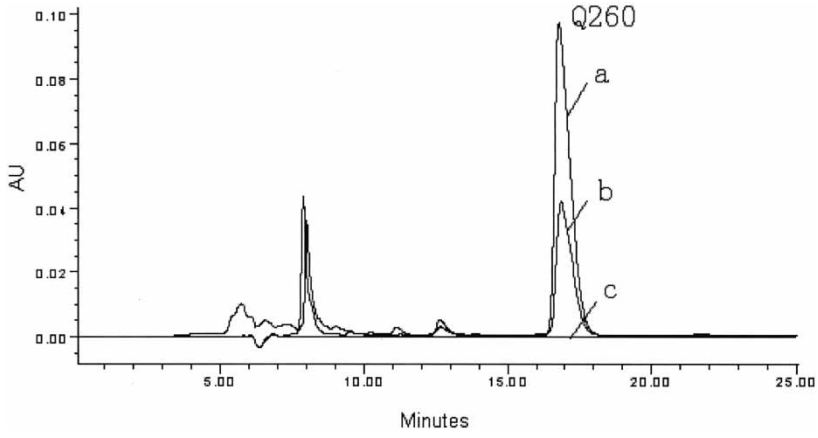

Figure 4. Chromatogram of sample solution detected at 260 nm. a: Q260 (standard solution, 104.66 μg/mL), b: sample solution (10.03 mg (herb)/mL), c: blank solution.

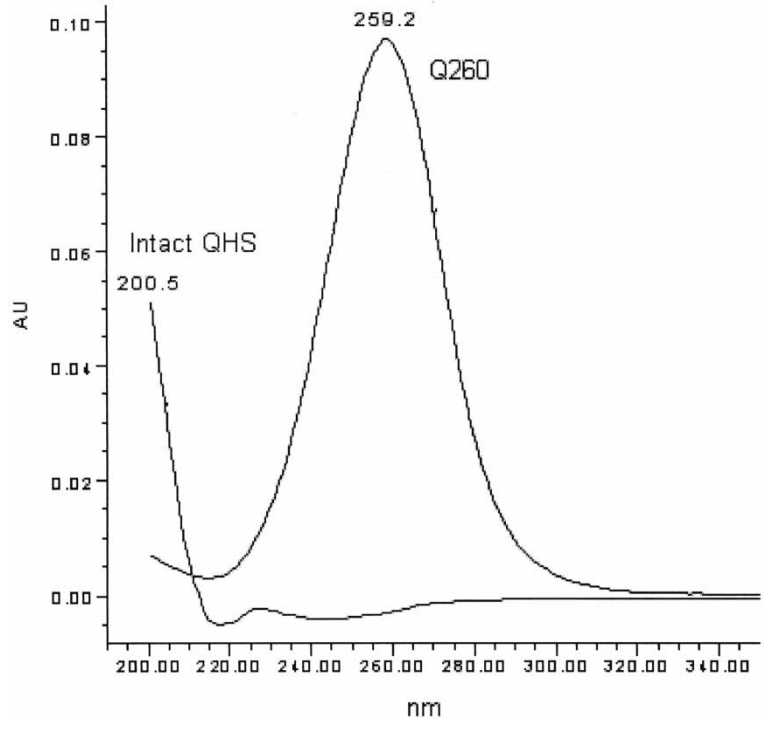

Figure 5. UV spectra of Q260 and intact QHS. Q260: 104.66 μg/mL in the mobile phase. Intact QHS: 1.046 mg/mL in the ethanol.

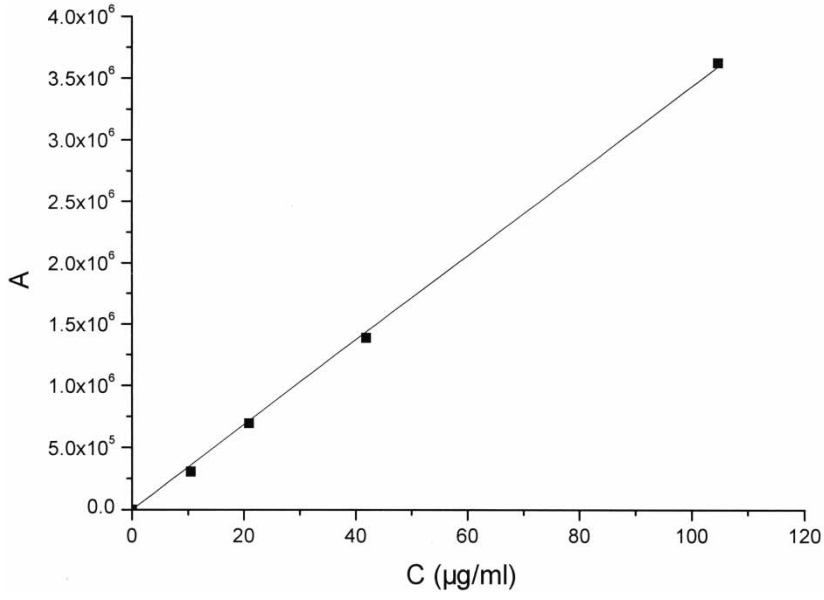

Figure 6. Calibration curve: peak area (Au · s) Vs concentration of artemisinin (μg/mL). Chromatographic condition: Column: C_{18} (250 mm × 4.6 mm i.d.). Mobile phase: 45/10/45 (by volume) methanol/acetonitrile/0.9 mM Na_2HPO_4-3.6 mM NaH_2PO_4 buffer (pH 7.76). Flow rate: 0.5 mL/min. 10 μL injected.

Assay Precision

Samples of *Artemisia annua L.* were divided into four aliquots. They were treated according to the preparation procedure of the sample, and each aliquot was analyzed three times by HPLC. The average artemisinin content of the herb is 0.652% and the relative standard deviation (n = 12) was 1.1%.

Analytical Recovery

Sigma artemisinin standard was added to the sample of *Artemisia annua L.*, then treated and analyzed by HPLC according to the procedure described above. The average recovery obtained was 98.9% and the relative standard deviation (n = 5) was 1.2%.

CONCLUSION

The high performance liquid chromatography with UV detection method has been improved and validated for the detection and quantification of

artemisinin in the herb of *Artemisia annua L.* The mobile phase was 45/10/45 (by volume) methanol/acetonitrile/0.9 mM Na_2HPO_4-3.6 mM NaH_2PO_4 buffer (pH 7.76). The artemisinin content in *Artemisia annua L.* was 0.652% according to this method, with relative standard deviation of 1.1%. The calibration curve was linear with an excellent correlation coefficient ($R = 0.9996$). The average recovery ($n = 5$) was 98.9% and the RSD was 1.2%. This method was accurate, sensitive, stable, reproducible, and suitable to routine analysis.

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