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Technical Note

Analysis of the Antimalarial Sesquiterpene Artemisinin in *Artemisia annua* by High-Performance Liquid Chromatography (HPLC) with Postcolumn Derivatization and Ultraviolet Detection

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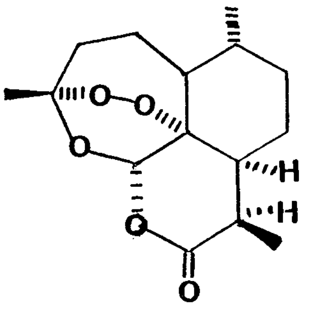
INTRODUCTION

Artemisinin (I), a sesquiterpene lactone with a peroxide function, is a novel antimalarial agent produced by the Chinese medicinal plant "qinghao" (*Artemisia annua* L. Asteraceae). Several reports for the quantitation of artemisinin appeared in the literature in the last few years. These include thin-layer chromatography (TLC) (1), high-performance liquid chromatography (HPLC) (2–7), and radioactivity measurement of the tritiated material (8).



Scheme I

The work described in this report deals with the quantitation of artemisinin in the leaves of *A. annua* using a modification of the procedure described by Edlund *et al.* (5). This assay was used to monitor the level of artemisinin in plant material in order to determine the best time for harvesting.

MATERIALS AND METHODS

Instrumentation. A Waters Model 590 solvent delivery system equipped with a postcolumn derivatization unit (Waters) was used. The detector was a LDC Milton Roy variable-wavelength detector set at 289 nm and an AUFS of 0.5.

Chromatographic Systems and Columns. The mobile phase was acetonitrile acetate buffer (55:45) at a flow rate of 0.45 ml/min. A 30 × 3.9-mm-i.d., C18 μBondaPak (10 μm) reversed-phase column (Waters Associates) was used together with a reversed-phase C18 guard column (15 × 3.2 mm, Brownlee Laboratory, Inc., Santa Clara, Calif.). After 150 injections, the guard column was changed, and the column regenerated by washing successively with 75 ml each of acetonitrile/water (1:1), acetonitrile, methanol/water (1:1), and methanol at a flow rate of 2 ml/min. The column was flushed at the end of each work day with 50 ml of acetonitrile/water (1:1, 2 ml/min) followed by acetonitrile (0.2 ml/min) overnight.

Postcolumn Reaction. A Teflon capillary (4.4 × 0.5 mm) was knitted according to Engelhardt and Neue (9) and kept wrapped on a test tube. The column and the knitted capillary tube were kept in a heated box at 70 ± 0.1°C through the use of a Waters temperature control module. The derivatizing reagent was 1 *M* KOH in methanol/water (9:1) at a flow rate of 0.2 ml/min. At the end of each working day the postcolumn derivatization pump was washed with 50 ml water, then 50 ml methanol by withdrawing 20 ml each time with a syringe, then pumping the solvent at 0.2 ml/min. When starting work, the mobile phase was allowed to pump at 2 ml/min for 10 min, then at 0.45 ml/min, and the derivatizing reagent at 0.2 ml/min. The system was left to stabilize for 45 min before making injections.

Analytical Standards. Artemisinin was isolated from *A. annua* L. Its purity was above 99% as determined by comparison (TLC, MMP, IR, ^{1}HNMR, MS) with an authentic reference sample obtained from the World Health Organization.

Chemicals. All solvents were HPLC grade and all other chemicals were reagent grade. Acetephenone was obtained from Aldrich.

Plant Material. Seeds of an Asian stock of *A. annua* were used. These were grown and collected from the medicinal Plant Garden, School of Pharmacy, University of Mississippi.

Acetate Buffer. The buffer solution with ionic strength

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(μ) = 0.1 and pH 5.1 was prepared by dissolving 8.3 g of sodium acetate and 4 ml of glacial acetic acid in HPLC-grade water to produce a final volume of 1 liter. All solutions were filtered and degassed under suction for 5 min using a filtration unit (Kontes Scientific Glassware/Instruments). These solvents were sonicated for 20 min prior to use.

Extraction Procedure. Artemisia leaves (10 g or less) were dried in an oven at 40°C for 24 hr, crushed, and extracted with hexane (100 ml) by boiling for 15 min under reflux. The hexane extract was evaporated to dryness at 40°C *in vacuo*. To the residue was added 25 ml of acetonitrile with sonication. An aliquot of the acetonitrile was filtered through a Millex HV 0.45-μm filtering unit (Millipore Corporation, Bedford, Mass.) and capped. Proper dilutions were made when necessary. Prior to injection, 100 μl of acetonitrile solution containing artemisinin was mixed with 100 μl of the internal standard (acetophenone, 1 mg/ml in acetonitrile), and from this solution 50 μl was injected.

RESULTS AND DISCUSSION

Artemisinin absorbs at the low end of the UV spectrum, between 210 and 220 nm. Although a method was developed for the quantitation of artemisinin using HPLC with UV detection at 210–220 nm (2,3), this detection at the low wavelength region has its obvious drawbacks, especially with plant extracts. Later, Zhao and Zeng (4) reported on the HPLC characteristics of the alkali-treated artemisinin (0.2% NaOH, heat in a water bath at 50°C for 30 min), which was found to have a UV absorbance at 292 nm. The modified artemisinin, thus obtained, was used for the quantitation of I in the petroleum ether extracts of the flowers and leaves of *A. annua* (4). A LiChrosorb RP-18 column and a mobile phase consisting of 0.01 *M* Na_2HPO_4/NaH_2PO_4 in MeOH/H_2O (45:55) were used.

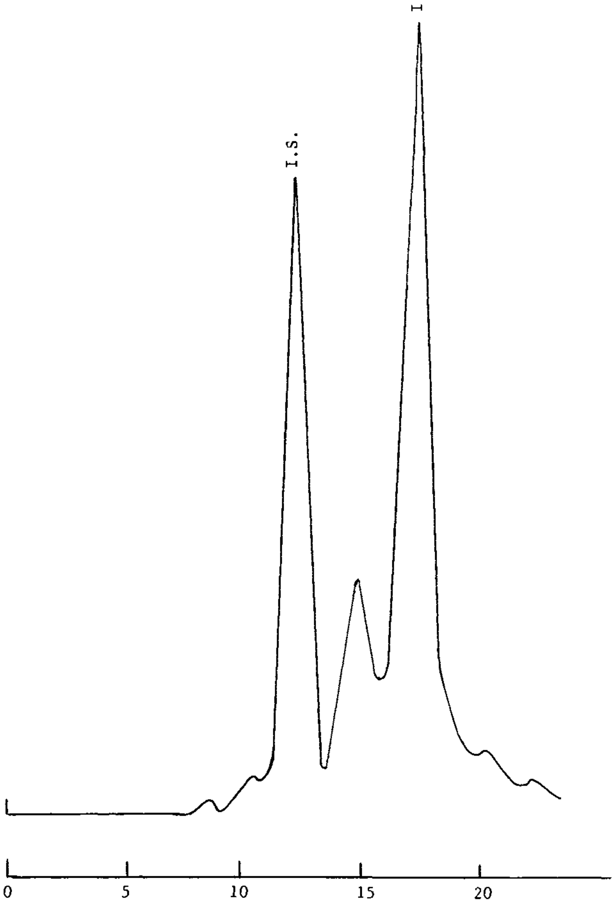

Fig. 1. Typical HPLC chromatogram obtained from a hexane extract of 19% *A. annua* showing acetophenone (I.S., at 11.5 min) and artemisinin (I, at 16.5 min).

Table I. Weekly Study to Determine Artemisinin Level (g/100 g Plant)

Date	Week	X ± SD	CV (%)
6/20	1	0.166 ± 0.0153	13.2
6/27	2	0.088 ± 0.028	31.2
7/3	3	0.113 ± 0.042	37.2
7/11	4	0.119 ± 0.046	38.6
7/18	5	0.087 ± 0.018	20.7
7/25	6	0.115 ± 0.043	37.4
8/1	7	0.153 ± 0.046	30.0
8/8	8	0.138 ± 0.026	18.8
8/14	9	0.140 ± 0.017	12.0
8/22	10	0.098 ± 0.039	39.8
8/31	11	0.108 ± 0.040	37.0
9/17	12	0.105 ± 0.034	32.4
9/14	13	0.067 ± 0.016	23.9

Based on the product obtained upon reacting artemisinin with alkali, Edlund *et al.* (5) reported on the determination of dihydroartemisinin and artesunate in plasma using HPLC. The same procedure, with modification, was used to analyze *Artemisia* leaves for artemisinin content using acetophenone as the internal standard. The knitted Teflon capillary column used in this work is a three-dimensional deformed tube in the shape of an eight, designed to minimize peak broadening during postcolumn derivatization (10–13). A representative chromatogram is shown in Fig. 1. The extraction procedure of the sequiterpene as described gave comparable results to Soxhlet extraction with the same solvent for 48 hr. Using the variable-wavelength detector set at 289 nm and an AUFS of 0.01, the minimum detection limit was 25 ng, which resulted in a signal-to-noise ratio of 3:1 with a coefficient of variation (CV) of ±10% (N = 10). A linear relationship was observed (r = 0.9995) between the peak height ratio and the concentration of artemisinin between 25 and 250 ng/ml.

The mean percentage recovery of artemisinin from plant material was calculated to be 89% (N = 5). *Artemisia vulgaris*, which is devoid of artemisinin, was spiked with artemisinin and the plant material extracted as above. Another portion of *A. vulgaris* was extracted and the resulting extract then spiked with the same amount of artemisinin. Recovery was calculated as the ratio of the apparent concentration of artemisinin in the first extract to that in the second extract.

A weekly study was initiated during the 1985 growing season in an attempt to find the best time for harvesting of *A. annua*. Sampling was carried out using five replicates each time. Results shown in Table I indicate that maximum amounts of artemisinin were reached prior to flowering (between 8/1/85 and 8/14/85). These values were 0.153, 0.138, and 0.140%, with a coefficient of variation of 30, 18.8, and 12%, respectively. Early flowering and full flowering result in a drop in the artemisinin content (0.105 and 0.067%, respectively). It is thus concluded that the procedure described in this report offers a reproducible and sensitive method for the analysis of artemisinin in plant material. In order to obtain the highest yield of artemisinin, the leaves should be harvested prior to flowering of the plants.

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