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A LARGE-SCALE EXTRACTION TECHNIQUE OF ARTEMISININ FROM *ARTEMISIA ANNUA*

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ABSTRACT.—An isolation method has been developed for the large scale production of artemisinin, a sesquiterpene lactone endoperoxide, from the leaves of *Artemisia annua*. The hexane extract of the unground dried leaves was partitioned between 20% aqueous MeCN and hexane (1:3). This partitioning step resulted in the quantitative transfer of artemisinin in the aqueous MeCN layer with concomitant reduction in the amount of material to 32–36% of the weight of the original hexane extract. Artemisinin was isolated from the aqueous MeCN fraction through chromatography on Si gel filtration columns. The method developed allowed the reuse of the recovered solvents and the chromatographic columns. Artemisinic acid and arteannuin B, two other sesquiterpenes, were also isolated.

Artemisinin, a novel sesquiterpene lactone endoperoxide, is becoming an important plant-derived compound in the treatment of the chloroquine-resistant and cerebral malarias (1). The World Health Organization has chosen β-arteether, the β-ethyl ether derivative of artemisinin, for the treatment of severe and complicated forms of *Plasmodium falciparum* malaria.[1] Thus, a need for adequate supplies of artemisinin is essential.

Very little information was found in the literature concerning the large scale isolation of artemisinin from the plant. Two procedures for the isolation of artemisinin (3,4) were reported. One of those procedures depends upon the use of the Ito-multilayer separator extractor (3). This procedure is only suitable for small scale extraction. The second procedure (4), while capable of isolating large quantities of artemisinin, suffers from several limitations which include: (a) the procedure depends upon chromatographing a relatively crude fraction on Si gel, which necessitates the use of a large ratio of adsorbent to solute; (b) the solvent system used in eluting the chromatographic column is 7.5% EtOAc in $CHCl_3$, resulting in the bulk of the eluting system being dense, expensive, and unstable; and (c) the order of elution when using such a system is artemisinin, arteannuin B, and artemisinic acid. As the acid is predominant it will tend to elute with artemisinin, and fractions containing artemisinin may require rechromatography to achieve the necessary purity. This work describes a practical and economic procedure for the isolation of artemisinin in kilogram quantities.

EXPERIMENTAL

PLANT MATERIAL.—Plants were grown from an Asian seed stock of *Artemisia annua* L. (Asteraceae). The leaves were collected in the preflowering stage from plants grown in the Medicinal Plant Garden, University of Mississippi. A voucher specimen has been deposited in the Herbarium, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi.

GENERAL EXPERIMENTAL PROCEDURES.—Melting points of the three sesquiterpene compounds were determined in an open capillary tube

[1]Scientific Working Group on the Chemotherapy of Malaria, 6-7 October 1986, WHO Headquarters, Geneva, Switzerland.

with a Thomas-Hoover capillary melting point apparatus, and are uncorrected. Ir spectra were recorded in KBr using a Perkin-Elmer 281B Infrared Spectrometer. Mass spectra were obtained using an E.I. Finnigan model 3200 (70 eV) with Incos data system. ^{1}H-nmr spectra were recorded on a Varian EM-390 90 MHz spectrometer using TMS as internal standard. ^{13}C-nmr data were taken on a JEOL FX60 Spectrometer operating at 15.03 MHz. Hplc analysis was carried out following the previously reported procedure (5).

CHROMATOGRAPHIC CONDITIONS.—Tlc chromatographic analyses were carried out on precoated Si gel G-25 UV254 plates (Macherey-Nagel Duren). The adsorbent used for cc was Si gel 60/170–270 mesh (Macherey-Nagel Duren). The developing system for tlc was 5–7.5% EtOAc/$CHCl_3$ solution, and visualization of the tlc plates was performed using anisaldehyde/H_2SO_4 spray reagent (6). The solvents, hexane, EtOAc, MeCN, and $CHCl_3$, were Fisher brand.

EXTRACTION.—*Laboratory Scale.*—Dried unground leaves (160 g) of *A. annua* were extracted with hexane in a Soxhlet for 48 h. The solvent-free extract (12 g, 7.8% hexane extractives) was partitioned three times between *n*-hexane (12 ml/g) and 20% aqueous MeCN (4 ml/g) presaturated with each other. The combined 20% aqueous MeCN was back-washed using 10% of its volume with presaturated hexane. Removal of H_2O from the MeCN phase was accomplished through the use of solid NaCl (7g/100 ml 20% aqueous MeCN). Evaporation of the MeCN in vacuo provided an oily yellowish-brown residue (4.31 g). Artemisinic acid was partially crystallized (420 mg) from this MeCN phase and removed prior to chromatography. Cc of the residue was conducted on Si gel 60 in the ratio of 1:10. The eluting system consisted of 10% EtOAc/hexane (1 column vol, 110 ml), followed by 15% EtOAc/hexane (1 column vol, 110 ml) and 20% EtOAc/hexane (1.5 column vol, 165 ml) at filtration flow rates (20 ml, 7 min.). Artemisinin was obtained in the column fractions eluted with 15% EtOAc/hexane (last 40 ml) and 20% EtOAc/hexane (1.5 column vol, 165 ml). Evaporation of the solvent produced 1.6 g of a greenish-yellow oil that crystallized readily from Et_2O-hexane (1:4). Recrystallization from CH_2Cl_2-hexane (1:4) afforded pure artemisinin (194 mg, 0.12% yield): mp 154° [lit. (4) 153–154°] mmp, ir, ^{1}H nmr, elemental analysis, and hplc identical in comparison with an authentic sample supplied by Dr. A. Brossi of the NIH, Bethesda, Maryland.

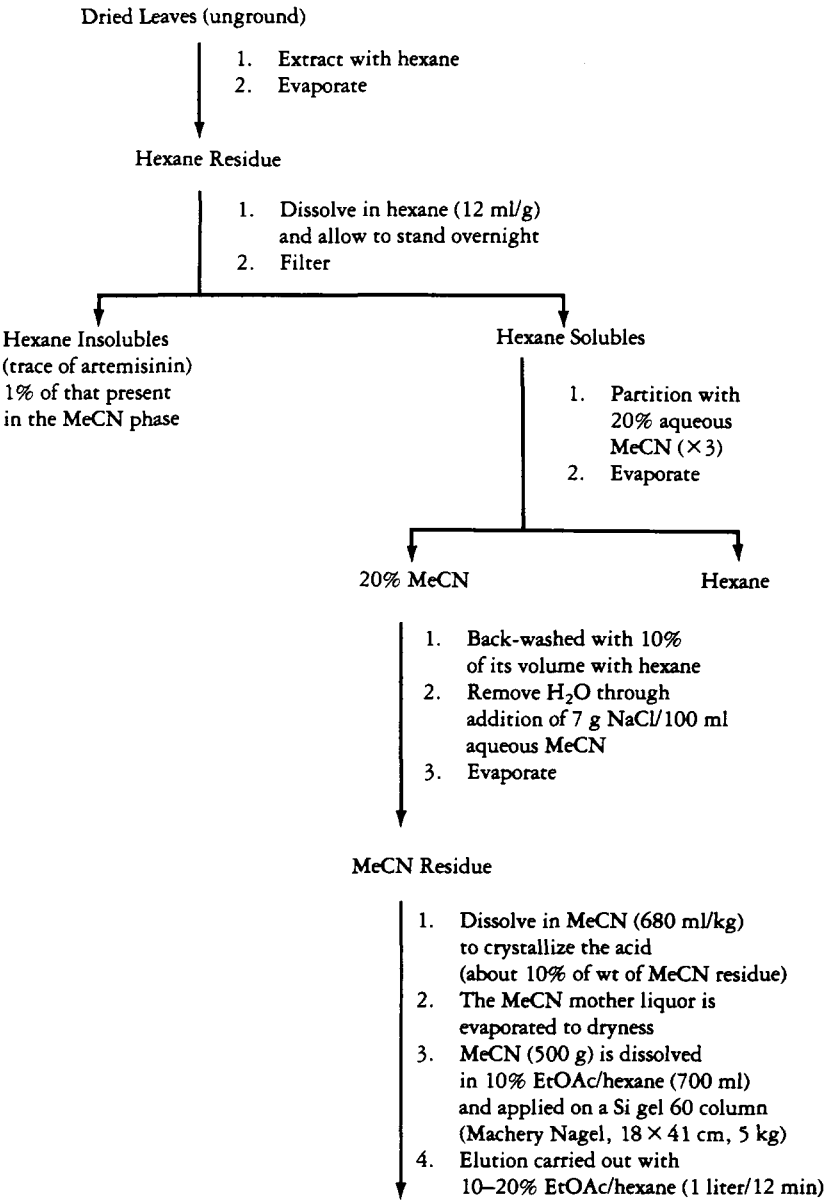
ISOLATION OF ARTEMISINIC ACID.—Artemisinic acid was isolated prior to artemisinin in the fraction eluted with 10–15% EtOAc/hexane (190 ml). Evaporation to dryness followed by crystallization from MeCN afforded colorless cubes (997 mg, 0.62%): mp 129–130° [lit. (7) 131°]. Ir, eims, ^{1}H- and ^{13}C-nmr spectral data were identical to those reported (7,8).

ISOLATION OF ARTEANNUIN B.—Arteannuin B was obtained in the fraction eluted with EtOAc (165 ml). Evaporation to dryness followed by crystallization from Et_2O-hexane (1:4) afforded rod-like colorless crystals (68 mg, 0.04%); mp 151–152°, [lit. (9) 152°]. Ir, eims, ^{1}H- and ^{13}C-nmr spectral data were identical to those reported (9–11).

Large Scale Isolation of Artemisinin.—Unground, dried leaves of *A. annua* (400 kg) were proportionally extracted, partitioned, and chromatographed as under laboratory scale (Figure 1). The weight of the soluble hexane extract was 29.2 kg (7.3%) and that of the MeCN phase 10.765 kg (36.8% that of the hexane extract). Artemisinic acid was partially crystallized from the MeCN residue (1.2 kg, using 680 ml MeCN/kg MeCN residue). The total amount of the isolated artemisinin from this batch was 485 g (0.12% yield), that of the acid was 2.12 kg (0.53%), and that of arteannuin B 170 g (0.04%).

RESULTS AND DISCUSSION

The procedure outlined in Figure 1 provides a simple, practical, and economical method for the large scale isolation of artemisinin from plant material. Leaves of *A. annua* were extracted with hexane followed by partitioning the hexane extract with 20% aqueous MeCN (3:1 ratio). This partitioning step resulted in quantitative transfer of artemisinin into the aqueous MeCN layer with concomitant reduction in the amount of material to be chromatographed. The use of other partitioning system, e.g., *n*-hexane/MeOH: H_2O (9:1), resulted in the distribution of artemisinin in both phases. Also, slight modifications of the ideal hexane/20% aqueous MeCN described under Laboratory scale and shown in example 1 of Table 1 showed reduction in the isolated yield of artemisinin. The yield of artemisinin was slightly better when the hexane phase was partitioned with 20% aqueous MeCN instead of MeCN (0.12% as opposed to 0.10%). Maximum yield of artemisinin was obtained (a) when the 20% aqueous MeCN phase was presatu-



1. Artemisinic acid (10–15% EtOAc/hexane 1.66 column vol, N.B. 1 column vol = 9 liters).
2. Artemisinin (15–20% EtOAc/hexane 1.88 column vol).
3. Arteannuin B (EtOAc wash 1.5 column vol).
4. Elution carried out with 10% EtOAc/hexane (9 liters) followed by 15% EtOAc/hexane (9 liters) and 20% EtOAc/hexane (13.5 liters).

FIGURE 1. Flow chart for isolation of artemisinin, artemisinic acid, and arteannuin B.

rated with the hexane phase; (b) when it was back-washed with 10% of its volume with hexane (presaturated with MeCN); and (c) when H_2O was removed as brine prior to evaporation.

The aqueous MeCN phase was chromatographed on Si gel columns using 10–20% EtOAc/hexane. Artemisinic acid, the major sesquiterpene, was eluted first with 10–15% EtOAc/hexane (15 liters). Artemisinin was obtained in the fraction eluted with 15–20% EtOAc/

TABLE 1. Effect of Slight Modification in the Hexane/20% Aqueous MeCN Partitioning System on the Yield of Artemisinin.

Example	Weight of Plant	Hexane Residue	Partitioning System	Weight of Phase II	Artemisinin Yield
1	160 g	12 g	Hexane (Phase I)/ 20% aqueous MeCN (Phase II) (presaturated with each other). Phase II back-washed with 10% of its volume with hexane, H_2O removed as brine	4.31 g	194 mg (0.12%)
2	160 g	12.3 g	Same as the above except phases not presaturated with each other.	4.05 g	137 mg (0.09%)
3	160 g	12.2 g	Same as above but Phase II directly evaporated without salting out the H_2O with NaCl	4.5 g	121 mg (0.08%)
4	160 g	12.5 g	Phases I and II as above except a) no saturation of phases b) Phase II not washed with 10% of its volume with hexane c) Phase II directly evaporated without removing the H_2O	4.6 g	111 mg (0.07%)
5	160 g	12.5 g	Hexane (Phase I) and nonaqueous MeCN (Phase II) presaturated with each other	6.2 g	160.2 mg (0.10%)

hexane (17 liters). Arteannuin B was eluted last in the EtOAc wash of the column (13.5 liters).

It is to be noted that the isolated artemisinin was >99% pure and was devoid of any artemisitene. The plant material used in this study was collected in the preflowering stage and showed no artemisitene by hplc analysis (5). This is in contrast to a previous report (12) of artemisitene/artemisinin ratios varying from 1:10 in early season to 1:1 as flowers develop.

The following are the major advantages of this process: (a) The partitioning step results in the selective transfer of artemisinin into the aqueous MeCN phase. (b) Almost 50% of the isolated artemisinic acid was directly crystallized from the MeCN phase prior to chromatography. (c) The solvent system used for chromatography allowed for faster migration of artemisinic acid than artemisinin; therefore the acid caused no interference with artemisinin. (d) The packing material in the columns was used in two runs. After each use, a quick rinse using 13.5 liters of EtOAc allowed for the reconditioning of the column. The polarity of the solvent system was decreased in the succeeding run to compensate for the partial deactivation of the Si gel. Therefore the solvent composition used in the second run was 8% EtOAc in hexane (9 liters), 13% EtOAc in hexane (9 liters), and 18% EtOAc in hexane (13.5 liters). The recovery of artemisinin from the reused column was comparable to that from a freshly packed column (93% based on a single determination). (e) The recovered EtOAc/hexane solvent mixture was used again after drying over anhydrous Na_2SO_4 (250 g per 3 liters) and adjusting its composition to the desired percentage level based on refractive index measurements. These advantages are of significant economic value for large scale isolation of this important antimalarial drug.

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