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Isolation and purification of azadirachtin from neem (*Azadirachta indica*) seeds using flash chromatography and high-performance liquid chromatography

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Azadirachtin ($C_{35}H_{44}O_{16}$), a tetranortriterpenoid from the neem tree (*Azadirachta indica* A. Juss), has generated wide academic (azadirachtin is hormonally active in insects[1,2]) and industrial (azadirachtin is currently being evaluated as a source[3] and a model[4] of new insect control agents) interests. The compound, and extracts containing it, have been found to have potent and specific effects against a variety of insect pests[5–8].

A preparative isolation technique for azadirachtin is needed to generate the significant quantities of the pure natural product needed for research purposes (its synthesis is not known). Unfortunately, the purification of azadirachtin is difficult to accomplish, especially on a preparative scale, due to the complexity and similarity-in-structure of the chemicals found in the seeds and foliage of the neem tree[9–11].

The best technique for the purification is high-performance liquid chromatography (HPLC)[12,13]. Other chromatographic techniques have been found to be inferior[14–17]. In this paper, we report on the preparative isolation of azadirachtin of single peak purity utilizing the rapid and inexpensive technique of flash chromatography[18] combined with HPLC.

MATERIALS AND METHODS

Extraction of azadirachtin from neem seeds

A suspension of 1.0 kg of ground neem seeds (obtained from India by Vikwood, Sheboygan, WI, U.S.A.) in 2.0 l of *n*-hexane was stirred occasionally at room temperature for several hours. The hexane extract was decanted and the process was repeated with fresh *n*-hexane three more times. The pooled hexane extracts were concentrated *in vacuo* to give 181 g of an orange solid. The defatted marc was then extracted six times with 2.0-l portions of methanol in the same manner as the *n*-hexane extractions. The combined filtered methanol extracts were concentrated *in vacuo* to give 78 g of an orange tar.

The orange tar was dissolved in 2.0 l of methanol and diluted by slowly adding, with stirring, 2.0 l of distilled water. This aqueous methanol mixture was then extracted three times with equal-volume portions of *n*-hexane followed by three equal-

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TABLE I

R_F VALUES OF AZADIRACHTIN IN VARIOUS SOLVENT SYSTEMS ON SILICA GEL AND OCTADECYLSILYL-SILICA GEL (ODS) ANALYTICAL TLC PLATES

Solvent system (v/v)	R_F	*TLC plate*
Diethyl ether–methanol (99:1)	0.24	Silica gel
Dichloromethane–acetone (4:1)	0.30	Silica gel
Diethyl ether–methanol (49:1)	0.37	Silica gel
Dichloromethane–methanol (19:1)	0.52	Silica gel
Diethyl ether–methanol–acetic acid (95:5:1)	0.55	Silica gel
Isopropanol–*n*-hexane (11:9)	0.56	Silica gel
Diethyl ether–acetone (2:1)	0.74	Silica gel
Acetonitrile–water (9:11)	0.32	ODS
Methanol–water (3:2)	0.37	ODS

volume portions of dichloromethane. Rotary evaporation *in vacuo* of the hexane layer, the aqueous methanol layer and the dichloromethane layer yielded 11.4 g of a yellow-brown oil, 44.6 g of a brown tar and 18.2 g of a dark orange amorphous solid, respectively.

Thin-layer chromatography (TLC)

Normal-phase analytical TLC was performed on 20 × 20 cm prescored silica gel GHLF plates (250 μm, Analtech) using one of seven solvent systems listed in Table I. Reversed-phase analytical TLC was performed on 1 × 3 in. $MKC_{18}F$ plates (200 μm, Whatman) using one of two solvent systems listed in Table I. Visualization for analytical TLC was accomplished under shortwave ultraviolet (UV) light, followed by spraying with a vanillin–sulfuric acid–ethanol (3 g:1.5 ml:100 ml) spray reagent and heating with a hot air gun.

Flash column chromatography

A 2.0-l flash column (Aldrich) was packed, after removal of fines, with silica gel (60–200 mesh, 15 × 7.0 cm I.D., J. T. Baker) and equilibrated with diethyl ether–methanol (49:1). The residue (18.2 g) from the dichloromethane extract described above was dissolved in 50 ml of dichloromethane and applied onto the top of the column. The column was then eluted with diethyl ether–methanol (49:1) at 25 ml/min into fractions of 20 ml. Fractions containing azadirachtin, as monitored by TLC (Table I), were pooled and rotary evaporated *in vacuo* yielding 7.4 g of an orange amorphous solid. This material was dissolved in 75 ml of methanol–water (3:2) and applied on to the top of a second flash column (2.0 l, Aldrich) packed with octadecylsilyl-silica gel (ODS) (40 μm, 15 × 7.0 cm I.D., Regis) in methanol–water (3:2) and applied onto the top of a second flash column (2.0 l, Aldrich) packed with of 20 ml. Fractions containing azadirachtin, again monitored by TLC, were pooled, azeotroped with isopropanol and rotary evaporated *in vacuo* to yield 1.26 g of a yellow solid.

High-performance liquid chromatography

Preparative HPLC was carried out with a Hewlett-Packard Model 1081B

liquid chromatograph. Samples were dissolved and injected onto the column using a Negretti and Zambra injector. Effluents were detected using a Micromeritics 787 variable-wavelength UV–VIS detector set at 254 nm and equipped with a micro-flow cell. Detected peaks and retention times were recorded using a Hewlett-Packard 3388A integrator.

The first of two preparative HPLC steps was done with a Phenomenex silica gel (particle size, 5 μm) stainless-steel column (25 × 2.0 cm I.D.) eluted isocratically with isopropanol–*n*-hexane (1:3) at a flow-rate of 5.0 ml/min and an average pressure of 1100 p.s.i. The column was protected with an Alltech stainless-steel guard column (5.0 × 0.46 cm I.D.) packed with Alltech pellicular silica gel. The second preparative HPLC step was performed on a Phenomenex phenyl (particle size, 5 μm) stainless-steel column (25 × 2.25 cm I.D.) eluted isocratically with acetonitrile–water (3:7) at a flow-rate of 5.0 ml/min and an average pressure of 1600 p.s.i. This column was protected with an Alltech stainless-steel guard column (5.0 × 0.46 cm I.D.) packed with Alltech pellicular phenyl.

Analytical HPLC was carried out on a Phenomenex phenyl (particle size, 5 μm) stainless-steel column (25 × 0.46 cm I.D.) using the same liquid chromatograph, corresponding guard column, detector (set at 218 nm) and injector system as described above. The solvent system consisted of acetonitrile–water (3:7) run isocratically at a flow-rate of 1.0 ml/min and an average pressure of 1000 p.s.i. Detected peaks were integrated (valley to valley) and retention times were recorded using a Hewlett-Packard 3388A integrator set at a peak width of 0.64, threshold of 0, attenuation of 2 and chart speed of 0.25 cm/min.

A second analytical HPLC method was carried out on a normal-phase Alltech silica gel (particle size, 5 μm) stainless-steel column (25 × 0.46 cm I.D.) eluted isocratically with isopropanol–*n*-hexane (1:3) at a flow-rate of 1.0 ml/min and an average pressure of 500 p.s.i.

Identification of azadirachtin

Azadirachtin isolated from neem seeds was identified by spectral comparison (IR, ^{1}H-NMR, fast atom bombardment mass spectrometry) and cochromatography (TLC, HPLC) with an authentic sample.

RESULTS AND DISCUSSION

The isolation of azadirachtin of >99% purity from neem seeds via extraction, partition, a combination of normal-phase and reversed-phase flash chromatography, and a combination of normal-phase and reversed-phase preparative HPLC runs is shown in Fig. 1. The extraction of azadirachtin with methanol was more efficient when the ground neem seeds were first defatted with *n*-hexane. To ensure complete recovery of azadirachtin, the defatted seeds were extracted six times with methanol at room temperature[19]. The crude azadirachtin was purified another four-fold by partitioning, first between methanol–water (1:1) and *n*-hexane, to remove small amounts of non-polar hexane-soluble impurities, and then between the aqueous methanol phase and dichloromethane. Azadirachtin was found by TLC (Table I) and HPLC to be confined to the dichloromethane fraction.

Even after the partition steps, the extract contained large amounts of pigments

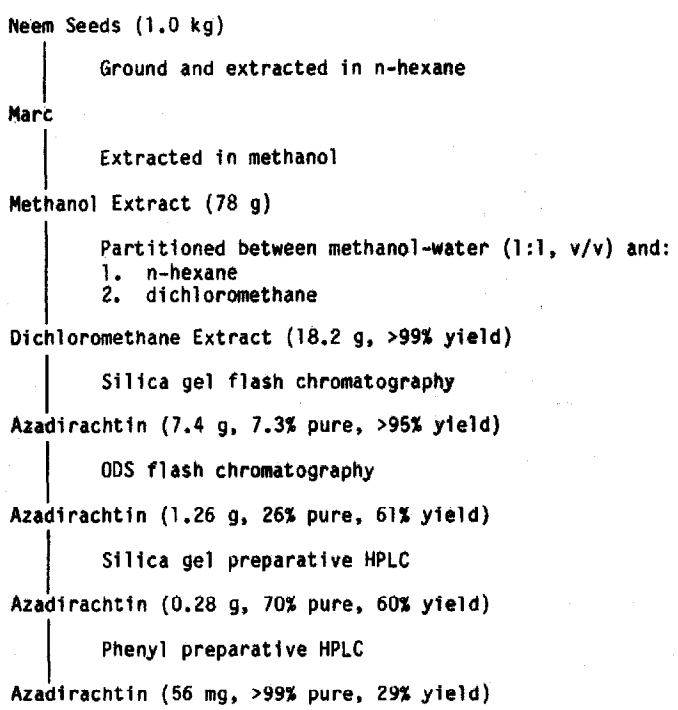

Fig. 1. Flow diagram of the isolation of azadirachtin from neem seeds.

and other impurities which adhered tenaciously to silica gel. Therefore, in order to prevent rapid contamination of the expensive HPLC columns, coarse (60–200 mesh) silica gel was used in the first flash chromatography step (Fig. 1).

Table I shows seven normal-phase and two reversed-phase analytical TLC solvent systems used to choose appropriate solvent systems for both flash chromatography and HPLC and to monitor the fractions from flash chromatography. A vanillin spray reagent was used to visualize azadirachtin on the TLC plates since it gave a characteristic color reaction of red-brown changing to green for azadirachtin.

The diethyl ether–methanol (49:1) and the methanol–water (3:2) solvent systems were chosen to be used in the purification of the neem seed extract on silica gel and ODS flash chromatography, respectively (Fig. 1), since azadirachtin moves on analytical TLC to an R_F of 0.37 in these solvent systems (Table I), close to the optimum (R_F = 0.35) for flash chromatography[18]. Each run on the flash column was capable of chromatographing gram quantities of material in less than 1 h. The two flash chromatography steps thus quickly gave, from a very complex mixture (Fig. 2), azadirachtin (26% pure) ready for easy purification by HPLC (Figs. 1 and 2).

Analytical HPLC was used to determine which fractions from preparative HPLC were appropriate for pooling. For reversed-phase HPLC, we found that aqueous acetonitrile achieved better resolution of azadirachtin in a shorter amount of time than did the previously reported aqueous methanol[16,19]. In addition, we

found the phenyl column to be superior to the previously reported ODS column[16,19]. The retention time (t_R) of azadirachtin in our system was 10.3 min.

A second analytical HPLC system (a normal-phase system) consisted of a silica gel column run isocratically at 1.0 ml/min with isopropanol–*n*-hexane (1:3). This system was chosen since it not only provided good separation of azadirachtin on normal-phase analytical TLC (Table I), but also because it did not interfere with the detection of azadirachtin at 218 nm. The other normal-phase TLC solvent systems listed in Table I were unsuitable for normal-phase HPLC because they were either too volatile (*viz.* diethyl ether) or had UV cut-off points at wavelengths greater than 218 nm (*viz.* acetone, dichloromethane). The t_R of azadirachtin in this normal-phase system was 13.5 min.

The two preparative HPLC methods used in this paper were chosen on the basis of the results obtained from analytical HPLC. The azadirachtin, prepurified by flash chromatography, was purified to 70% by silica gel preparative HPLC (Fig. 2). We found this step necessary to remove a minor impurity not well separated from azadirachtin by preparative HPLC on a phenyl column. Typically, each injection, containing 500 mg of 26% pure azadirachtin in 0.8 ml of methanol, yielded 110 mg of 70% pure azadirachtin. The approximate t_R for azadirachtin in this system (see above) was 35 min.

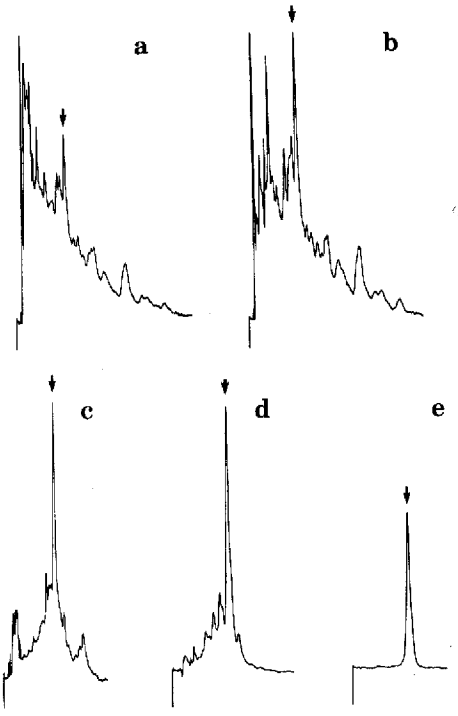

Fig. 2. Analytical high-performance liquid chromatograms of azadirachtin (arrows) recovered after: (a) dichloromethane extraction; (b) silica gel flash chromatography; (c) ODS flash chromatography; (d) silica gel preparative HPLC; (e) phenyl preparative HPLC. Analytical HPLC was performed with a phenyl column (25 × 0.46 cm I.D., 5 μm particle size) eluted isocratically with acetonitrile–water (3:7) at 1.0 ml/min.

The final purification step utilizing preparative HPLC on a phenyl column gave, in each run, azadirachtin of >99% purity (*i.e.* single peak purity by analytical HPLC) (Fig. 2). Each injection, containing 400 mg of 70% pure azadirachtin in 0.8 ml of acetonitrile, typically gave 81 mg of >99% pure azadirachtin. The approximate t_R for azadirachtin in this reversed-phase system (see above) was 53 min. The lower yield (29%) obtained in the final purification step was due to the conservative manner in which the fractions were pooled (to assure complete homogeneity of azadirachtin) and represented a minimum for this step. By rechromatographing the other fractions containing azadirachtin, as much as 64% combined yield was obtained.

Flash chromatography is a rapid, inexpensive and easily performed technique[18] with a large sample capacity. The fact that only single runs were necessary in each preparative HPLC step was made possible by the prepurification of the neem seed extract by flash chromatography.

For some of our investigations absolutely pure azadirachtin is not a necessity. For example, in our structure–activity relationship studies, chemically modified derivatives of azadirachtin have to be purified from their reaction mixtures. In such cases, where >85% pure azadirachtin is adequate, the silica gel preparative HPLC step can be deleted from the overall isolation scheme.

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REFERENCES

1 G. Käuser and J. Koolman, in W. Engels, W. H. Clark, Jr., A. Fischer, P. J. W. Olive and D. F. Went (Editors), *Advances in Invertebrate Reproduction 3*, Elsevier, Amsterdam, New York, 1984, p. 602.
2 E. S. Garcia and H. Rembold, *J. Insect Physiol.*, 30 (1984) 939.
3 R. O. Larson, personal communication.
4 Z. Lidert, personal communication.
5 R. C. Saxena, in L. W. Shemilt (Editor), *Chemistry and World Food Supplies: The New Frontiers*, Pergamon, New York, 1983, p. 143.
6 M. F. Balandrin, J. A. Klocke, E. S. Wurtele and W. H. Bollinger, *Science (Washington, D.C.)*, 228 (1985) 1154.
7 H. Schmutterer, K. R. S. Ascher and H. Rembold (Editors), *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss). Proc. 1st Int. Neem Conf. (Rottach-Egern, 1980)*, German Agency for Technical Cooperation, Eschborn, 1981.
8 H. Schmutterer and K. R. S. Ascher (Editors), *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss) and Other Tropical Plants. Proc. 2nd Int. Neem Conf. (Rauischholzhausen, 1983)*, German Agency for Technical Cooperation, Eschborn, 1984.
9 D. Lavie, E. C. Levy and M. K. Jain, *Tetrahedron*, 27 (1971) 3927.
10 I. Kubo, A. Matsumoto, T. Matsumoto and J. A. Klocke, *Tetrahedron*, (1986) in press.
11 W. Kraus, in Cs. Szántay, A. Gottsegen and G. Kovács (Editors), *Chemistry and Biotechnology of Biologically Active Natural Products. Proc. 2nd Int. Conf. (Budapest, 1983)*, Elsevier, Amsterdam, New York, 1984, p. 331.
12 H. Rembold, H. Forster, Ch. Czoppelt, P. J. Rao and K.-P. Sieber, in H. Schmutterer and K. R. S. Ascher (Editors), *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss) and Other Trop-*

ical Plants. Proc. 2nd Int. Neem Conf. (Rauischholzhausen, 1983), German Agency for Technical Cooperation, Eschborn, 1984, p. 153.
13 H. Rembold, in W. Engels, W. H. Clark, Jr., A. Fischer, P. J. W. Olive and D. F. Went (Editors), *Advances in Invertebrate Reproduction 3*, Elsevier, Amsterdam, New York, 1984, p. 481.
14 J. H. Butterworth and E. D. Morgan, *J. Insect Physiol.*, 17 (1971) 969.
15 P. R. Zanno, I. Miura, K. Nakanishi and D. L. Elder, *J. Am. Chem. Soc.*, 97 (1975) 1975.
16 E. C. Uebel, J. D. Warthen, Jr. and M. Jacobson, *J. Liq. Chromatogr.*, 2 (1979) 875.
17 H. Rembold, G. K. Sharma, Ch. Czoppelt and H. Schmutterer, *Z. Pflanzenkr. Pflanzenschutz*, 87 (1980) 290.
18 W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 43 (1978) 2923.
19 J. D. Warthen, Jr., J. B. Stokes and M. Jacobson, *J. Liq. Chromatogr.*, 7 (1984) 591.