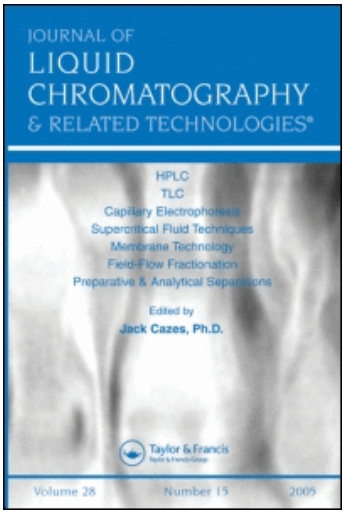

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Isolation of Various Azadirachtins from Neem Oil by Preparative High Performance Liquid Chromatography

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ISOLATION OF VARIOUS AZADIRACHTINS FROM NEEM OIL BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Isolation of various azadirachtins, ie., Azadirachtin A,B, D, H and I in pure form from neem oil by preparative high performance liquid chromatography procedure is described.

INTRODUCTION

In a recent publication in this journal we had reported the isolation of the major constituents of neem oil (oil from the seeds of *Azadirachta indica* A.Juss.),[1] like salannin, nimbin, deacetylnimbin, azadiradione and epoxyazadiradione by direct preparative high performance liquid chromatography using a reverse phase column. However, the first two peaks (peak 1 and peak 2 in ref[1]) in these preparative runs gave relatively small amounts of material and the compounds present were not isolated although some of them were identified and quantitated, such as azadirachtin A, B, D, H

and I by analytical HPLC. In a very recent publication[2] HPLC analysis of neem oil using a semi-preparative reverse phase column has been carried out and a few major triterpenoids like nimbandiol, nimbanol, salannin have been identified by HPLC-MS procedure and quantified by the mass intensities, but the compounds were not isolated.

Herein, we report the actual isolation of various individual azadirachtins present in neem oil by preparative HPLC procedure.

MATERIALS AND METHODS

Preparative High Performance Liquid Chromatography was carried out using a Shimadzu LC8A HPLC system linked to CR4A data processor and the peaks detected at 215 nm. Two Shimpack reverse phase (C_{18}) preparative columns (25 cmx50 mm i.d.) and (25 cm x 20 mm i.d.) were used for preparative runs and Phenomenex reverse phase column (C_{18}) (25 cm x 4.6 mm) was used for analysis.

Neem oil, (1 lit) obtained by using a cold mechanical expeller was partitioned between n-hexane and 90% methanol and the methanol extract was concentrated to dryness *in vaccuo* at 45° (62.8g). This was subjected to preparative HPLC for the isolation of major triterpenoids.

RESULTS AND DISCUSSION

For each preparative run 5 g of the above residue from methanol extract was dissolved in 20 mL of methanol, filtered through a Millipore filter (0.25μm) and then injected into the preparative column (20 cm x 50 mm i.d). The eluent flow rate was 30 mL/min throughout the run.

During the first sixty minutes 60:40 MeOH:H_2O was used and the more polar compounds like azadirachtins were eluted. From 60 minutes the eluent was changed to 70:30 MeOH:H_2O and atleast six major peaks eluted out. The identities of the compounds in these six peaks have already been described.[1]

We report here the actual isolation of various azadirachtins from the peak-1 (rt. 34.3 min.) and peak-2 (rt. 43.7 min.)[1] obtained during the first sixty minutes by subjecting them to further preparative high performance liquid chromatography.

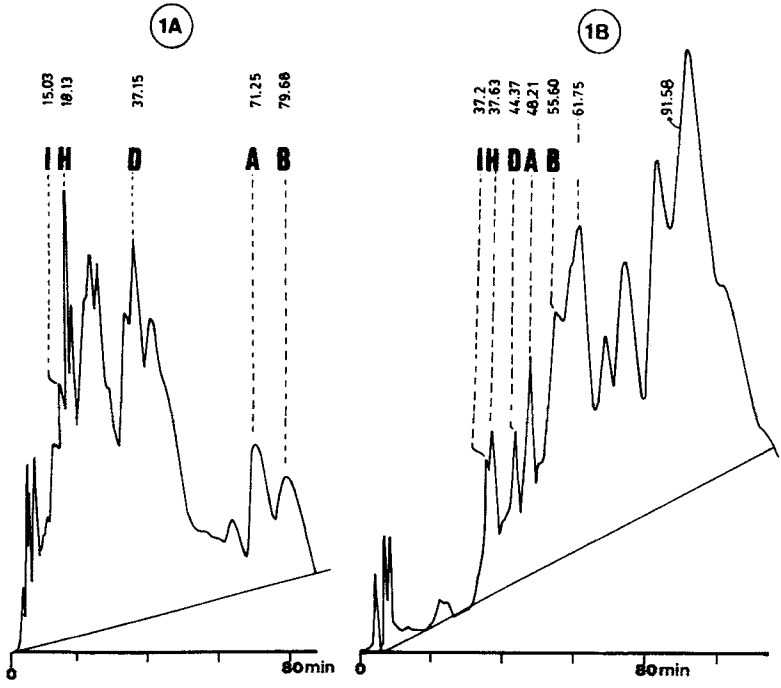

Figure. 1A Preparative High Performance Liquid Chromatogram of Peak-1
Figure. 1B Preparative High Performance Liquid Chromatogram of Peak-2

Peak-1 (3g, 500mg/4 mL MeOH for each run) was subjected to preparative high performance liquid chromatography using 25 cm x 20 mm i.d. column with 28:72 CH_3CN:H_2O as eluent at 12 mL/min flow rate (Fig. 1A). The individual peaks were collected and evaporated to yield various azadirachtins, the identity of these were established by analytical HPLC using 25 cm x 4.6 mm i.d. column with 15 : 35: 50 CH_3CN : MeOH : H_2O as eluent at 1mL/min flow rate and comparing the retention times with authentic azadirachtin samples.

Thus peaks with retention times 15.03 min. (132 mg), 18.13 min.(264 mg), 37.15 min.(310 mg), 71.25 min. (180 mg) and 79.68 min.(300 mg) were identified to be pure Azadirachtin I, Azadirachtin H, Azadirachtin D, Azadirachtin A and Azadirachtin B respectively (Table 1).

Table 1

Isolation of Various Azadirachtins from Peaks 1 and 2 in Neem Oil

Sl. No.	Compound	mg of Compound		Total	% Isolated from Neem Oil
		Peak 1	Peak 2		
1	Azadirachtin I	132	56	188	0.0188
2	Azadirachtin H	264	168	432	0.0432
3	Azadirachtin D	310	100	410	0.0410
4	Azadirachtin A	180	99	279	0.0279
5	Azadirachtin B	300	172	472	0.0472

Pk-2 (3.5 g , 500mg/4 mL MeOH for each run) was subjected to preparative high performance liquid chromatography using 25 cm x 20 mm i.d. column with 28:72 CH_3CN : H_2O as eluent and 15 mL/min flow rate (Fig. 1B). The individual peaks were collected and evaporated to yield various azadirachtins, the identities of these were done by analytical HPLC as indicated for peak 1.

Thus peak 2 yielded Azadirachtin I(56 mg), Azadirachtin H (168 mg), Azadirachtin D (100 mg), Azadirachtin A (99mg) and Azadirachtin B (172 mg) respectively (Table 1).

In peak-2 two more peaks with retention times of 61.75 min. (48 mg) and 91.58 min.(165 mg) were collected, but the identities of these compounds have yet to be established. The identity and purity of the individual azadirachtins were further confirmed by spectral data and comparison with standard samples by analytical HPLC.

It is evident that peak 1 (of reference 1) contains more of the polar compounds like the azadirachtins and peak 2 also furnishes the same azadirachtins albeit in smaller amounts. The reason for the cross over of these compounds to peak 2 (reference 1) could be the high rate of elultion (30 mL/min) and the greater eluting capacity of the solvent employed (60:40 MeOH : H_2O). Peak 2 also contains more of the less polar compounds, whose identities have yet to be established.

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