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Short Communication

Quantitation of azadirachtins in insecticidal formulations by high-performance liquid chromatography

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ABSTRACT

A sensitive high-performance liquid chromatographic procedure for the quantitative determination of azadirachting A and estimates of other azadirachtins in insecticidal formulations is described. The procedure utilizes reversed-phase chromatography with detection by UV absorption at 215 nm. The quantitative method described has been found to be accurate to within ± 10% relative between the concentrations of 0.005 and 0.75% (w/w) azadirachtin A in insecticidal formulations of neem kernel extract. The lower limit of detection is 0.001% (w/w).

INTRODUCTION

Azadirachtin is a limonoid of the tetranortriterpenoid type found in the neem (*Azadirachta indica*) and chinaberry (*Melia azedarach*) trees [1,2]. The Indian neem tree is a fast growing robust tree found throughout India, Pakistan and parts of Africa as well as other parts of the world (*e.g.*, Australia and Haiti) [3]. Nearly the entire tree —roots, leaves and fruits— can potentially be used for agricultural, industrial and commercial products [4]. In particular, formulations made from neem kernel extract hold promise for use as plant protection products.

Since its isolation by Butterworth and Morgan [5], much world-wide interest, both academic and industrial, has developed in azadirachtin and related compounds due primarily to its powerful antifeedant and growth-disruptive activity toward a variety of insect pests [6,7]. In fact, azadirachtin is one of the most powerful naturally occurring insect-feeding deterrents known [8] from a botanical source. In spite of its powerful insecticidal activity, azadirachtin is non-mutagenic [9] and does not appear to exhibit any mammalian toxicity [10,11]. With increasing awareness of the hazards of chemical insecticides, the availability of a safe, natural product for insect and pest control is very attractive.

Azadirachtin is only one of many tetranortriterpenoids present in neem kernel which exhibit some degree of insecticidal properties. Others include salannin [12], nimbidin [13], and meliantriol [14]. Nevertheless, azadirachtin appears to be the most active of any of the limonoids studied. Recent work has demonstrated that azadirachtin itself should be designated azadirachtin A, since there exist at least six other compounds isolated from neem kernels that are structurally very similar to azadirachtin (designated B–G) [7,15]. Commercial products made

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from seed extract, then, will have a multitude of natural products present in addition to azadirachtin A. Formulations from neem kernels may contain additional constitutents such as triglycerides, fatty acids and surfactants which can interfere with chromatographic analysis. A rapid and reliable method for determining azadirachtin A concentration is needed in order to determine the quality and potency of such complex insecticidal formulations.

Several HPLC methods have described reversed-phase methodology for the purification or analysis of azadirachtin [15–19] as well as an supercritical fluid chromatography (SFC) method [20]. However, these methods have been applied to relatively "clean" matrices at the end of an isolation procedure or have used a technique such as SFC which is not routine instrumentation. No reports have described the routine separation of azadirachtin A from azadirachtin B in insecticidal formulations. Here we present a sensitive and rugged HPLC method capable of quantifying azadirachtin A in stable, commercial formulations of neem kernel extract.

EXPERIMENTAL

Reagents and materials

Acetonitrile, methanol and tetrahydrofuran (THF) were from Baker (Phillipsburg, NJ, USA). Eluents were prepared with water purified by a Milli-Q water purification system (Millipore). Solid-phase extraction (SPE) tubes (C_{18}, 3 ml, 500 mg adsorbant) were from Baker and used without conditioning. Azadirachtin A was isolated essentially according to the procedure of Schroeder and Nakanishi [21]. From 11 kg of neem kernels, approximately 8 g of 77% pure azadirachtin A were isolated. Approximately 500 mg of this were further purified by preparative HPLC to 200 mg of >95% purity. The purity of azadirachtin A was determined using quantitative NMR and optical rotation measurements [22].

Equipment

The chromatographic system consisted of Waters Model 510 pumps, a WISP autosampler, Model 680 gradient controller and Model 480 variable-wavelength detector; all from Waters Associates (Milford, MA, USA). Data collection and handling was accomplished using LCI-100 integrators and a Chromatographics 3 Data System (Perkin-Elmer, Norwalk, CT, USA). A Supelcosil RP-8 column (15 cm × 4.6 mm, 3 μm)(Supelco, Bellefonte, PA, USA) with a guard column (2 cm, 40 μm) was used for all reversed-phase work and the azadirachtins were monitored at 215 nm. The neem components were eluted isocratically with acetonitrile–water (28:72, v/v) followed by a column wash using THF–acetonitrile (90:10). A 10-μl injection size was used at a flow-rate of 1.0 ml/min. For preparative-scale work, a Supelcosil RP-8 column (25 cm × 2.5 cm) was used.

The NMR spectra were collected with a Bruker AC 300 or AM 400. Azadirachtin was prepared in deuterated chloroform or deuterated methanol. ^1H NMR spectra were collected with a relaxation time of 15 s. Azadirachtin spectra were referenced against two internal standard spikes of quinoxiline and trimethoxybenzene for the purposes of quantitation.

Standard preparation

Azadirachtin A was prepared at approximately 1.0 mg/ml concentrations in methanol and stored at −20°C. This preparation was found to be stable for at least 6 months. From this stock standard appropriate dilutions are made into methanol to cover the concentration range 0.010 to 0.150 mg/ml azadirachtin A. These calibration standards are stable for at least 2 months when kept refrigerated.

Sample preparation

Samples of the insecticide formulation (Margosan-O Botanical Insecticide Concentrate) were prepared by carefully weighing (± 0.1mg) 500 μl of a vortexed aliquot into 9.5 ml of methanol–water (90:10). This diluted sample was then vortexed for 30 s before application of 2 ml to a C_{18} SPE tube (unconditioned) in order to retain unwanted materials which fouled the HPLC column. The liquid was eluted at < 2 ml/min, the first 0.5 ml was discarded and the remainder was dispensed into an autosampler vial.

Different sample diluents for SPE were evaluated to determine optimal retention of column fouling compounds (triglycerides) while maintaining 100% azadirachtin A recovery. SFC was employed to detect triglyceride levels in the SPE filtrates. A metha-

nol–water (90:10) mixture resulted in greater than 98% adsorption of triglycerides and fatty acids with full recovery of azadirachtin A and B.

Internal standards were occasionally used in storage stability studies. Several candidates were evaluated including benzyl alcohol, phenyl ethanol and phenyl propanol. Phenyl propanol was judged to be the most suitable internal standard based on its elution in an area free of other components.

RESULTS AND DISCUSSION

The two major challenges associated with the development of this chromatographic procedure were the separation of azadirachtin A from azadirachtin B and the effective gradient washing of trigylcerides, fatty acids and other components from the reversed-phase column. This washing procedure resulted in substantial extension of the column lifetime.

Chromatographic separation

Since a multitude of other limonoids and natural products are present in neem kernel extract, an efficient chromatographic method is required to obtain resolution of azadirachtin A from these other constitutents. The nearest eluting neighbor to azadirachtin A was azadirachtin B as confirmed by liquid chromatography–mass spectrometry of both standards and formulations. Initial chromatographic separations did not resolve the A and B azadirachtins. Several different columns were tested including C_{18} and C_8 materials from different manufacturers. Several different blends of methanol, acetonitrile and tetrahydrofuran with water were examined. Modifiers such as trifluoroacetic acid and acetic acid were examined as well as buffered mobile phases. The conclusion was that a 15 cm 3 μm C_8 column (Supelco) was found to give the best resolution between azadirachtin A and B with an acetonitrile-water mobile phase. Though a 25 cm column (5 μm) would give slightly better resolution, the equilibration time after the wash would have incurred a long turnaround time for each analysis ($>$ 50 min). The shorter column provided adequate resolution with a minimum turnaround time. Though the method is reliable and rugged, care must be taken when preparing the mobile phase as a small error in acetonitrile concentration gave a significant shift in azadirachtin A retention.

Since the chromatographic run was isocratic and the insecticidal formulation contains significant amounts of triglycerides and other components, carryover peaks were observed in subsequent chromatographic runs. To alleviate this problem, a suitable washing procedure was required. After investigation of several organic modifiers including methanol, isopropanol and acetonitrile, THF was found to be most effective. After elution of the azadirachtins (typically 19–24 min) the THF mobile phase was introduced and held for 5 min before returning to the acetonitrile–water (28:72). After an equilibration time of 10 min, the next injection was made. Total run time was thus 38 min. The THF washing protocol resulted in extending the column lifetime from an average of 150 injections to over 2000 injections. Representative chromatograms of purified azadirachtin A and of formulation containing azadirachtin A and B are shown in Fig. 1.

Estimation of concentration of azadirachtin B and other limonoids

Based on the structures of azadirachtin A and B and other limonoids such as salannin, one can estimate that the extinction coefficients of these compounds are similar. Chromatographic separations using a photodiode array detector revealed that the spectra for azadirachtin A and B were very similar. Most of the contribution to absorbance at 215 nm is from the α,β-unsaturated carbonyl chromophore in the tiglate ester and the vinyl ether. For limonoids that have similar types and numbers of such moieties this estimation should be reasonable. By assigning an extinction coefficient to azadirachtin B equivalent to that of azadirachtin A, one can generate estimates of the concentration of azadirachtin B. A similar procedure can be used to estimate other identifiable azadirachtin analogs and limonoids whose structures are sufficiently similar to that of azadirachtin A.

Precision and recovery

Precision was determined by analysis of six preparations of a neem kernal extract formulation. The precision of the method was 3.5% relative standard deviation.

Recovery was determined by spiking two preparations of the formulation at two different levels of azadirachtin A before analysis. Formulations con-

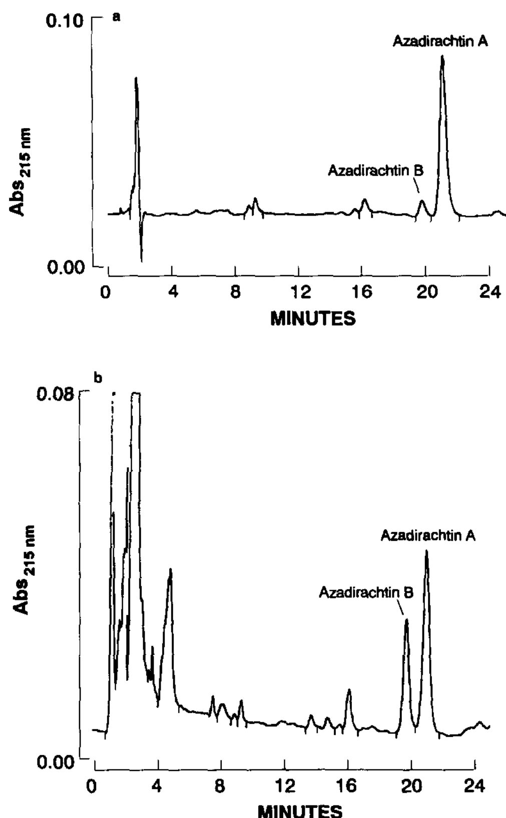

Fig. 1. (a) HPLC separation of purified azadirachtins A and B. The injection represents 0.08 mg/ml of azadirachtin A. (b) HPLC separation of azadirachtins A and B in Margosan-O. The injection represents a 50 mg/ml preparation of the formulation and 0.07 mg/ml of the azadirachtins.

taining approximately 0.126 mg/ml azadirachtin A were spiked at levels of 0.164 mg/ml and 0.302 mg/ml. Recovery ranged from 93 to 106%.

CONCLUSIONS

A procedure for the accurate quantification of azadirachtin A in insecticidial formulations has been presented. In addition, an estimation of azadirachtin B concentration can be derived from the method. The chromatography gives excellent resolution of azadirachtin A from azadirachtin B. The method is rugged and accurate and capable of quantifying azadirachtin A in the complex matrix containing oils, surfactants and other limonoids.

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