ELSEVIER

Journal of Chromatography A, 705 (1995) 374–379

JOURNAL OF CHROMATOGRAPHY A

Short communication

Determination of azadirachtin by reversed-phase high-performance liquid chromatography using anisole as internal standard

R. Thejavathi, Shirish R. Yakkundi, B. Ravindranath*

Vittal Mallya Scientific Research Foundation, P.O. Box 406, K.R. Road, Bangalore 560 004, India

First received 18 October 1994; revised manuscript received 22 February 1995; accepted 6 March 1995

Abstract

An accurate and convenient method of quantitation of the well-known biopesticide azadirachtin by reversed-phase high-performance liquid chromatography has been developed, using anisole as the internal standard. The method is precise and accurate within limits of ± 1.0% in the concentration range of 0.00002% (w/v) to 0.004% (w/v) of azadirachtin in solution (0.2–40 ppm) or 2–400 ng by weight of azadirachtin (10 μl injection; 20 ng of internal standard). Application of the method to stability studies of azadirachtin showed that the compound is reasonably stable (95–99%) in a variety of common solvents at room temperature (7 days) and to ultrasound (30 min). An efficient sample preparation protocol has been developed giving a recovery of 99.5 ± 4%.

1. Introduction

Azadirachtin (also known as azadirachtin A; Fig. 1) is a tetranortriterpenoid (limonoid) present in neem seeds (*Azadirachta indica* A. Juss) to amounts of 0.2 to 0.6% [1]. The neem tree, which yields about 30–40 kg/year of the seeds, is widely distributed in South Asian and several other tropical countries. Azadirachtin has gained world-wide attention for its insect antifeedant and ecdysis inhibiting properties [2,3]. This compound is highly potent at low concentrations against more than 200 agricultural pests and it is ecofriendly [4]. Thus, it has the potential to be a safe alternative to the toxic synthetic pesticides and a number of commercial formulations are being introduced world-wide. However, wide application of azadirachtin as a pest control agent requires a sensitive and reliable method for its quantitation in neem extracts, commercial formulations and foods, for monitoring its efficacy, stability and toxicity.

Azadirachtin occurs in neem together with nearly 100 other limonoids including azadirachtins B–K with closely related structures and its separation and quantitation is a challenging task. Reversed-phase high-performance liquid chromatography has been conveniently used for separation and quantitation of azadirachtin. The known methods of quantitation of azadirachtin generally use an external standardization method [4,5]. The main drawback of these methods is the inaccuracy in azadirachtin determination due to the possible loss of the compound during sample preparation. Such problems in quantita-

* Corresponding author.

0021-9673/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)00314-2

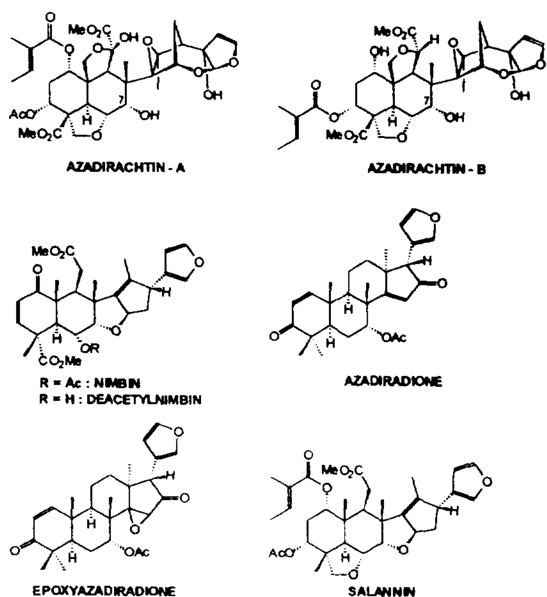

Fig. 1. Structures of major limonoids from *Azadirachta indica* A. Juss.

tion can generally be overcome by using an internal standard.

Quantitation by the internal standard method is sensitive and reproducible as a known amount of internal standard is added to the sample early in the sample preparation protocol and the compound is analysed as a ratio with respect to the internal standard. In the most recent paper on the HPLC analysis of azadirachtin by Hull et al. [7], benzyl alcohol, phenylethanol and phenylpropanol have been suggested as possible internal standards but without supporting data. None of these compounds are satisfactory because of the large differences in the chromatographic and spectral properties between the analyte and the standards. Also, the accuracy is only ± 10% in the range of 0.005 to 0.75% which is not sufficient for monitoring azadirachtin in the concentration ranges in which it is effective. Thirdly, the HPLC system used (3 μm Supelcosil RP-8 column, 150 × 4.6 mm I.D.) requires a 40-min run time and an elaborate column washing protocol with tetrahydrofuran. Therefore, the method is not very convenient for routine use.

In this paper, we report a fast, sensitive and accurate method for the quantitation of azadirachtin by using anisole as the internal standard. The efficacy of the method is supported by recovery studies and the method has been used to determine the stability of azadirachtin in different solvents.

2. Experimental

2.1. Chemicals and materials

Anisole from SD Fine Chemicals Pvt. (Bombay, India) was used without further purification. Solvents were obtained from E. Merck (India) (Bombay, India), and were distilled before use. Water used was deionised and purified using a Milli-Q water purifier from Millipore (Bedford, MA, USA). Azadirachtin was isolated from neem seeds by the method described by Schroeder and Nakanishi [8]. A 50-mg amount of azadirachtin (90% pure) thus obtained was subjected to preparative HPLC to yield 99% pure compound which was used to prepare calibration curves. Neem seeds were collected from Nagallur, a village in Andhra Pradesh, India.

2.2. Equipment

A Model 2100 UV spectrophotometer from Shimadzu Corporation (Kyoto, Japan) was used for spectroscopic studies. The HPLC system used consisted of two Model 510 reciprocating pumps, a Model U6K loop injector, a Model 484 tunable wavelength absorbance detector at 217 nm, a Maxima 820 FC system controller and data processor, a Novapak C_{18} (4 μm, 150 × 3.9 mm I.D., for analytical chromatography) and μBondapak C_{18} (10 μm, 300 × 10 mm I.D., for preparative purpose), all from Waters Associates (Milford, MA, USA); the sonicator (VC 600 Watt model dual output) was obtained from Sonics and Materials (Danbury, CT, USA), and the Remi centrifuge (Model R23) from Remi Sales (Bombay, India). The Whatman 3-piece filter funnel with glass microfibre filters (2.5-cm)

was from Whatman International (Maidstone, UK).

2.3. Chromatographic procedure

The solvent program used for complete analysis of neem extract was an isocratic elution with acetonitrile–water (40:60, v/v) for 5 min followed by a linear gradient to 100% acetonitrile in 3 min (20% increase per min) with a total run time of 12 min including a preacquisition delay of 2 min to eliminate the solvent peak in quantitation experiments. For preparative HPLC, 40% aqueous acetonitrile was used as the mobile phase with a flow-rate of 4.4 ml/min.

2.4. Standard solutions

Standard solution of azadirachtin (0.1%) was prepared by dissolving 50 mg of the compound in 50 ml of methanol. An anisole stock solution of 2 mg/ml was made and later diluted 100-fold to obtain a concentration of 20 μg/ml. A 1-ml volume of this solution was mixed with a calculated volume of azadirachtin solution and made up to 10 ml to cover the concentration range 0.2–100 μg/ml, with an anisole concentration of 2 μg/ml. Three 10-μl aliquots of each mixture were injected onto the HPLC system and the area and height responses determined using the data processor. For stability studies, the anisole stock solution (2 mg/ml) was diluted 10-fold to yield a concentration of 200 μg/ml.

2.5. Sample preparation

A 5-g amount of the powdered solid sample (e.g. neem seed powder) was sonicated (18 mm horn; 50% duty cycle; amplitude 5) for 30 min with methanol (12.5 ml) containing 250 μg of anisole. The mixture was centrifuged and the supernatant decanted. The solid was mixed with 8 ml of methanol and centrifuged again. The supernatants were pooled in a 25-ml volumetric flask and made up to the mark with 50% aqueous methanol to bring the total concentration of methanol in the test solution to 90% aqueous methanol. The solution was passed through a Whatmann three-piece filter funnel. A 1-ml volume of the filtrate was passed through a C_{18} Sep-Pak cartridge ("Classic", volume = 1 ml, 400 mg of ODS) and eluted with 3 ml of 90% aqueous methanol. The eluants were pooled and made up to 5 ml in a volumetric flask with 90% aqueous methanol and a 10-μl aliquots was injected onto the HPLC column (Fig. 2).

2.6. Recovery studies

De-oiled neem seed powder was obtained by extracting 100 g of neem seed powder with chloroform (500 ml) in a percolator. A 5-g amount of the powder was analysed for azadirachtin content as described above (0.005%). A 50-g amount of the neem seed powder (containing 2.5 mg of azadirachtin) was added to 100 ml of dichloromethane containing 10 mg of azadirachtin. Dichloromethane was evaporated and the dry powder was used for recovery studies the following day. A 5-g amount of the neem seed powder was subjected to the sample preparation protocol given above.

2.7. Stability of azadirachtin

To determine the stability of azadirachtin in different solvents, approximately 2.5 mg of azadirachtin was dissolved in the organic solvent (chloroform, acetone, ethyl acetate, acetonitrile, methanol and ethanol) and made up to the mark in a volumetric flask (10 ml) with the same solvent. A 1-ml volume of this solution was immediately mixed with 1 ml of anisole solution (200 μg/ml) in a 10-ml volumetric flask and made up with methanol, and a 10-μl aliquot was analysed on the HPLC system. A 1-ml volume of each of the solutions was refluxed for 8 h and taken in a 10-ml volumetric flask containing 1 ml of anisole stock solution and made up with methanol as before. The remainder of the solution was kept at room temperature for 7 days and analysed as before. Stability of azadirachtin to the sample preparation protocol was checked by dissolving 1.0 mg azadirachtin in methanol (10 ml) containing anisole (1 mg) and determin-

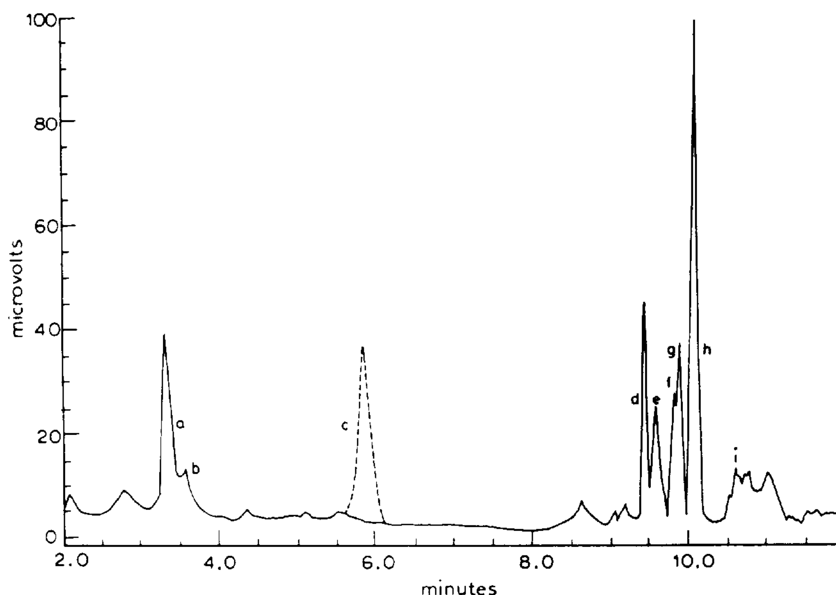

Fig. 2. Chromatogram of the neem seed extract. For sample preparation and chromatographic conditions, see text. Peaks: a = azadirachtin A; b = azadirachtin B; c = anisole (internal standard); d = deacetylnimbin; e = unidentified; f = azadiradione; g = nimbin; h = salannin; i = epoxyazadiradione.

ing the ratio of azadirachtin to internal standard before and after 30 min sonication in methanol.

3. Results and discussion

A variety of reversed-phase columns based on octadecylsilyl (C_{18}) and octylsilyl (C_8) stationary phases have been used for separation of neem limonoids. These columns show a wide range of selectivities and efficiencies. In the present study, we have used a Waters Novapak C_{18} column (150 × 3.9 mm I.D.) which, we have found, is particularly efficient for the separation of azadirachtin A and azadirachtin B ($\alpha = 1.1$), the main active constituents of neem, as well as the other major limonoids. Chromatographic analysis of a neem seed extract using a water–acetonitrile gradient required less than 12 min (Fig. 2).

Choice of an internal standard for azadirachtin was indeed difficult. An ideal internal standard should have similar chromatographic and spectral properties as the compounds to be analysed but should not normally co-occur with them nor co-elute with any of the peaks in the analyte. Though structurally very different from azadirachtin, we have found that anisole is a very good candidate as internal standard for azadirachtin, the reasons being: (a) the $A_{1\%}$ of anisole at 217 nm is 233, which is comparable to that of azadirachtin (173); (b) the separation factor of anisole to azadirachtin is comfortably close (around 1.8); (c) anisole is stable and neutral in nature and does not react with azadirachtin or other components of the sample; (d) the ratio of azadirachtin to anisole remained constant through the sample processing protocol; (e) anisole does not interfere with other components of the sample as shown by the chromatograms of neem extract with anisole in Fig. 2; and (f) the suitability of anisole as internal standard is further proved by recovery studies. It may also be mentioned that because of the different ranges of UV absorption (λ_{max} at 205 nm and 280 nm, respectively) and its appearance in the middle of the chromatogram (Fig. 2), well separated from both polar and nonpolar limonoids, anisole could also be the internal standard for several of the other important limonoids of

neem, the structures of which are shown in Fig. 1.

3.1. Calibration curve for azadirachtin with anisole as internal standard

The minimum detectable amount of azadirachtin (defined at a signal-to-noise level of 3) was 2 ng (10-μl injection volume). The response ratio of azadirachtin to anisole, taking either area or height, was found to be linear within the concentration ratio 0.1–20. Thus, the calibration curves for the response of azadirachtin with respect to anisole were obtained with the concentration range of azadirachtin being 0.2–40 μg/ml with anisole at 2 μg/ml (10-μl injection volume). Data was processed on Maxima 820 FC system controller and data processor. The linear regression analysis ($n = 33$) of the calibration curves yielded the following equations (a) for areas, $y = 0.413x - 0.053$ (y = area response ratio, x = mass ratio) with the standard error for slope and intercept being 0.002 and 0.015 (correlation coefficient $r = 0.9996$), respectively; (b) for heights, $y = 0.52x - 0.041$ (y = height response ratio, x = mass ratio) with the standard error for slope and intercept being 0.002 and 0.015, respectively, with correlation coefficient $r = 0.9998$. Beyond the linear range, the slope of the plot deviated appreciably from the above values. Thus, at an anisole-to-azadirachtin ratio of 1:25 the deviation from the slope was $\pm 2.5\%$ and at 1:30, it was $\pm 5\%$. For concentrations beyond the upper limit, i.e. 40 μg/ml, and up to 1 mg/ml, it was found more accurate to use the internal standard at a concentration of 20 μg/ml, the equations being $y = 0.32x + 0.015$ and $y = 0.43x + 0.024$ for area and height ratios, respectively. The standard errors are 0.004 and 0.011 (height ratio) and 0.003 and 0.010 (area ratio) for slope and intercept, respectively.

3.2. Precision and accuracy

For the determination of precision and accuracy of the method, a sample of known concentration of azadirachtin (0.0016%) was analysed using slopes of the calibration curves (both by area and height ratio). The precision (relative standard deviation = 100 × standard deviation/mean, $n = 3$) was $\pm 0.95\%$ and $\pm 1.8\%$ considering height and area ratios, respectively. Accuracy (measured concentration × 100/actual concentration) was 100.1% when the height ratio was considered and 98.9% when the area ratio was used. Thus, use of the height ratio gives more accurate results than the area ratio for lower concentrations of azadirachtin.

3.3. Stability of azadirachtin

Azadirachtin is known to be susceptible to light and variation in pH. It is also said to be unstable in solution. Knowledge of the stability of azadirachtin would help in developing methods for its extraction, separation and formulations for use as a pesticide as well as for developing reliable analytical protocols. The stability of azadirachtin was therefore examined in six common organic solvents.

Azadirachtin (2.5 mg) was dissolved in different solvents, i.e. chloroform, acetone, ethyl acetate, acetonitrile, methanol and ethanol (10 ml each; azadirachtin concentration, 0.025% w/v). A 1-ml volume of each of these solutions was mixed with 1 ml of anisole solution (200 μg/ml) and the detector response ratio of azadirachtin to anisole at zero time was determined. The azadirachtin concentration in the different solvents was determined in a similar manner after 7 days at room temperature and indicated that azadirachtin was reasonably stable in all solvents tested (95–99%). The azadirachtin concentration after 8 h of reflux in the same solvents indicated that azadirachtin was less stable to higher temperatures (90–95%). For sample preparation purposes (see below), the stability of the azadirachtin to anisole ratio was also determined before and after sonication for 30 min in methanol (100%; $n = 5$).

3.4. Sample preparation and recovery

As noted earlier, the types of samples in which azadirachtin may need to be determined is quite varied. They may be solids (example; neem

seeds) or solutions with a variety of diluents, requiring different methods of sample preparation. For liquid samples Hull et al. [7] have suggested dilution with 90% aqueous methanol followed by solid-phase extraction using appropriate reversed-phase columns to remove the lipids prior to injection onto the HPLC columns.

For solid samples, rapid extraction by sonication in methanol was found to be efficient. The suitability of using anisole as internal standard has been demonstrated by recovery studies. Five samples of de-oiled neem seed powder, spiked with a known amount of azadirachtin (1 mg/5 g), were subjected to the sample preparation protocol and analysis as given above. Azadirachtin was determined using the calibration curves. The recovery (mean ± S.D.) was 99.5 ± 4% considering height ratios. It may be mentioned that since the neem seed powder used in the experiment was de-oiled by extraction with chloroform, it contained much less of azadirachtin (5 mg% compared to 0.25% w/w in the original seeds) and other limonoids than those reflected in Fig. 2.

Acknowledgements

We acknowledge the financial support from International Development Research Centre, Canada. We also thank Dr. P.R. Krishnaswamy, Scientific Director V.M.S.R.F., and Prof. P.V. Subba Rao, Indian Institute of Science, Bangalore, for encouragement and support.

References

[1] T.R. Govindachari, G. Sandhya and S.P.G. Raj, J. Nat Prod., 55 (1992) 596.

[2] J.H. Butterworth and E.D. Morgan, Chem. Comm., (1968) 23.

[3] J.D. Warthen, Jr., *Azadirachta indica*: a source of insect feeding inhibitors and growth regulators, USDA, Sci. and Educ. Adm. Agric. Reviews and Manuals, Northeastern Ser. 4, Beltsville, MD, 1979, p. 21.

[4] A.J. Mordue and A. Blackwell, J. Insect Physiol., 39 (1993) 903.

[5] J.D. Warthen, Jr., J.B. Stokes, M. Jacobson and M.F. Kozempel, J. Liq. Chromatoqr., 7 (1984) 591.

[6] K.M.S. Sundaram and J. Curry, J. Liq. Chromatogr., 16 (1993) 3275.

[7] C.J. Hull, Jr., B.S. Switzer and W.R. Dutton, J. Chromatogr., 633 (1993) 300.

[8] D.R. Schroeder and K. Nakanishi, J. Nat. Prod., 50 (1987) 241.