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STRATEGY FOR THE PREPARATIVE-SCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION OF KADSURENONE AND FUTOQUINOL FROM THE MEDICINAL PLANT *PIPER FUTOKADSURA*

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SUMMARY

The chemical constituents of a Chinese herbal plant, haifenteng (*Piper futokadsura*), have been investigated. In addition to terpenes and lignans of diverse chemical structure, a potent receptor antagonist of platelet activating factor (PAF), kadsurenone, has been isolated and identified. PAF is a highly potent lipid mediator of acute inflammation, allergy and anaphylaxis. Its receptor antagonists are potential therapeutic agents for asthma and various cardiovascular and inflammatory disorders. More recently, a plant sample from Taiwan was re-examined in detail by normal-phase open column and high-performance liquid chromatography. The co-elution of sigificant amounts of futoquinol, a known lignan previously isolated from *P. futokadsura*, hampered the isolation of kadsurenone. To facilitate the isolation of kadsurenone a separation of futoquinol and kadsurenone from a methylene chloride extract of plant was developed on 10-μm silica using gradient chromatography with hexane–methylene chloride–ethyl acetate–acetonitrile. Silica packings of 10, 15 and 37–55 μm were evaluated with this gradient and the 15-μm silica was selected for the initial fractionaton of plant extract. The kadsurenone-containing fractions from the normal-phase separation were re-chromatographed using reversed-phase chromatography. A 15-μm C_{18} column using water–methanol and water–acetonitrile mobile phases yielded pure kadsurenone.

INTRODUCTION

The chemical constituents in the stems of *Piper futokadsura* (haifenteng), a medicinal plant found in southeast China, have been examined with numerous terpenes and lignans described[1–4]. The isolation of a novel receptor antagonist of platelet activating factor (PAF), kadsurenone (I, Fig. 1), has generated renewed interest in this medicinal plant[5]. PAF is a highly potent mediator of acute inflammation, allergic reactions, anaphylaxis and ischemia[6,7]. PAF receptor antagonists are potential therapeutic agents for the treatment of asthma and various cardiovascular and inflammatory disorders. Kadsurenone has been isolated from

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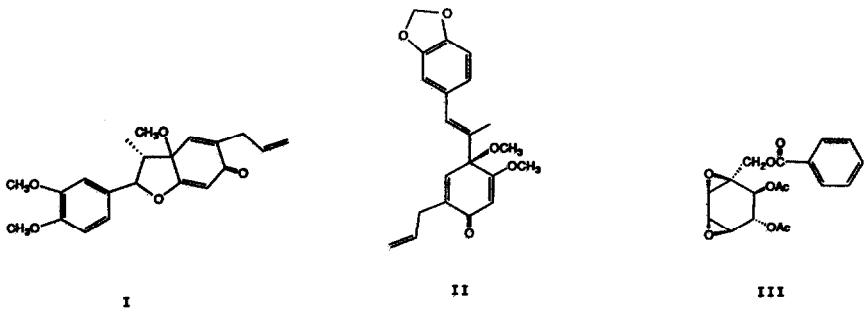

I II III

Fig. 1. Structures of kadsurenone (I), futoquinol (II) and futoxide (III). Ac = Acetyl.

methylene chloride extracts of haifenteng by open column chromatography on silica gel using hexane with increasing amounts of ethyl acetate. Fractions containing kadsurenone were further purified on 55–100-μm particle silica with a mobile phase of hexane–ethyl acetate (3:1, v/v)[5].

Recent attempts to obtain kadsurenone from a haifenteng sample collected in Taiwan, using this separation scheme, have been hampered by the presence of significant amounts of two lignans identified as futoquinol (II, Fig. 1) and futoxide (III, Fig. 1)[8]. Both lignans have been prevously isolated from *P. futokadsura*[9,10]. Additionally, kadsurenone can only be crystallized from very concentrated solutions and the amount of kadsurenone in this recent extract is low compared to previous plant extracts examined. To facilitate the isolation of kadsurenone from this extract for further chemical and biological studies, a more practical and effective preparative high-performance liquid chromatographic (HPLC) procedure was developed.

EXPERIMENTAL

Materials

Solvents and reagents used were of HPLC or reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Water was purified through the Millipore reverse-osmosis and cartridge filtration system (Millipore, Bedford, MA, U.S.A.).

A Taiwan specimen of *Piper futokadsura* was a gift from the Merck, Sharp and Dohme Research Labs. (Rahway, NJ, U.S.A.). A 1-kg amount of the stems of *P. futokadsura* was extracted with 2 l of methylene chloride for 3 days. After removal of the methylene chloride 20 g of extract were obtained; 12 g were used in this study. The crude methylene chloride extract was prepared in the laboratories of Dr. T. Y. Shen, Department of Chemistry, University of Virginia (Charlottesville, VA, U.S.A.).

Normal-phase chromatography

Analytical and preparative normal-phase chromatography was performed on the Delta-Prep instrument equipped with a Model 481 variable-wavelength UV detector set at 290 nm with a semi-preparative flow cell (Waters Chromatography Division of Millipore, Milford, MA, U.S.A.). For analytical chromatography 10 mg of crude extract, dissolved in 150 μl of methylene chloride, were separated on a 30 cm $\times$ 0.39 cm I.D. column packed with 10-, 15- or 37–55-μm silica (Waters). Eluents, (A) hexane, (B) methylene chloride, (C) ethyl acetate and (D) acetonitrile, were used at

a flow-rate of 1 ml/min to generate the following gradient: A–B (25:75) for 5 min, a 5-min linear gradient to 100% B followed by a 5-min hold, a 20-min linear gradient to B–C–D (59:37:4), and a 10-min gradient to C–D (96:4) to wash the column. The same gradient was used to separate 1, 5 and 6.5 g of crude extract, dissolved in 30, 150 and 200 ml of hexane–methylene chloride (25:75) on a 30 cm × 4.7 cm I.D. Prep Pak cartridge packed with 15-μm silica (Waters). The flow-rate was 80 ml/min and the samples were applied to the column through a port on the solvent delivery system. Fractions (1 min) were collected from all separations. Fractions from the preparative separations were brought to dryness and weighed. Futoquinol in fractions 28 and 29 from the 5- and 6.5-g preparative separations (2 g) was crystallized using cold methanol.

Reversed-phase chromatography

The equipment used for the analytical reversed-phase (RP) HPLC was two Model 510 pumps, a WISP multiple sample injector and a Model 440 UV detector set at 254 nm. Data analysis and system control were provided by a Model 840 chromatography work station (Waters). Aliquots (1 ml) of the fractions from the normal-phase separations were dried and re-dissolved in 1 ml of methanol. Volumes of 5 or 10 μl were analyzed on a 15 cm × 0.39 cm I.D., 5-μm Delta Pak C_{18} column with isocratic elution of water–methanol (35:65) at a flow-rate of 0.5 ml/min. The analyses and spectral comparison of the isolated futoquinol and kadsurenone to standards were made on the same system equipped with a Model 990 photodiode-array UV detector set to scan from 240 to 400 nm.

Preparative RP-HPLC of the kadsurenone-containing fractions from the normal-phase separation was performed on a Model 600 HPLC instrument, with a Model 490 multi-wavelength detector, set at 340 and 290 nm, and a Model 840 chromatography work station (Waters). Fractions 30 (1.2 g), from the 5- and 6.5-g normal-phase separations, were each dissolved in 3 ml of methanol. A white crystalline precipitate, futoxide, was removed and 1.0 or 1.5 ml (approximately 300 mg) of supernatant, containing kadsurenone, was injected on a 30 cm × 1.9 cm I.D., 15-μm Delta Pak C_{18} column. The kadsurenone was isolated using isocratic elution with water–acetonitrile (55:45) at a flow-rate of 5 ml/min. Six preparative separations were required to process all the material. Fractions 31 (0.6 g), from the 5- and 6.5-g normal-phase separations, were each dissolved in 1.5 ml of methanol, and the precipitate was removed. Six 1-ml injections of the supernatant (approximately 70 mg) were applied to the 15-μm Delta Pak C_{18} column and eluted with water–methanol (40:60) at a flow-rate of 5 ml/min.

RESULTS AND DISCUSSION

Since standards of both futoquinol and kadsurenone were available, a number of separation techniques were explored. Futoquinol and kadsurenone were easily separated on RP-HPLC C_{18} columns using either water–acetonitrile or water–methanol gradients. Resolution of these two compounds from the crude extract was identical to the standards. In addition RP-HPLC separated kadsurenone from the other plant constituents. RP-HPLC isolation of kadsurenone directly from extract, however, proved impractical for large-scale separations, because many of the

compounds present in the crude extract had limited solubility in the aqueous methanol or acetonitrile mobile phases. Attempts to put even moderate amounts of extract (mg loads) caused a substantial increase in the operating pressure of the column. The column could be cleaned and returned to normal operating pressures only after extensive washing with acetonitrile, which would be impractical for preparative-scale chromatography. The removal of insoluble and low-solubility components from the crude extract by selective precipitation always resulted in co-precipitation of kadsurenone.

Due to the limited solubility of futoquinol and other plant constituents in reversed-phase eluents, the development of a normal-phase HPLC method to separate futoquinol and kadsurenone was undertaken. Analytical-scale RP-HPLC was effective in assessing the purity of fractions collected from subsequent normal-phase separations. Isocratic elution with water–methanol (35:65) on a 5-μm, 15 cm × 0.39 cm I.D. Delta Pak C_{18} column resolved many of the plant constituents. Since kadsurenone was soluble in reversed-phase eluents, preparative HPLC was used for its purification from fractions collected from normal-phase chromatography.

Normal-phase chromatography on a 30 cm × 0.39 cm I.D., 10-μm silica column showed that using any combination of hexane–ethyl acetate mobile phases co-eluted futoquinol and kadsurenone. Methylene chloride–ethyl acetate gradients resolved the two compounds; however, the limited resolution of futoquinol and kadsurenone restricted the amount of extract that could be injected. Separation of futoquinol and kadsurenone was greatly improved by holding the initial conditions, hexane–methylene chloride (25:75), for 5 min followed by a 5-min gradient to 100% methylene chloride. These conditions were held for 5 min to elute the futoquinol and then kadsurenone was eluted by a 5-min gradient to methylene chloride–ethyl acetate (80:20). The resolution of kadsurenone from the other plant constituents was maximized as shown in Fig. 2A, by replacing the gradient to methylene chloride–ethyl acetate (80:20) with a 20-min gradient to methylene chloride–ethyl acetate–acetonitrile (59:37:4).

Using the gradient conditions presented in Fig. 2, 10 mg of extract, dissolved in methylene chloride, were injected on 30 cm × 0.39 cm I.D. columns packed with 10-, 15- and 37–55-μm silica as shown in Fig. 2. Analyses of these fractions using the reversed-phase method, described previously, showed the futoquinol to be well separated from kadsurenone (not shown). RP-HPLC analyses of the kadsurenone isolated from both the 10-μm (not shown) and 15-μm silica columns eluted relatively free of other plant constituents as shown in Fig. 3. However, RP-HPLC analysis of kadsurenone isolated from the 37–55-μm silica showed the presence of contaminants (Fig. 4); therefore, 15-μm silica was chosen as the packing for the preparative-scale separations.

A loading study was carried out to determine the maximum amount of extract that could be applied to the 15-μm column. More than 65 mg of crude extract showed loss of resolution between futoquinol and kadsurenone, while 10 to 45 mg of extract yielded 75–80% of the kadsurenone, contaminated with one major component. The sample mass for a fixed resolution applied to a preparative column is a function of the cross-sectional area and can be increased as the squares of the diameters of the columns[11]. Using a scale factor of 145 increases the preparative sample load to between 1.45 and 6.5 g of crude extract.

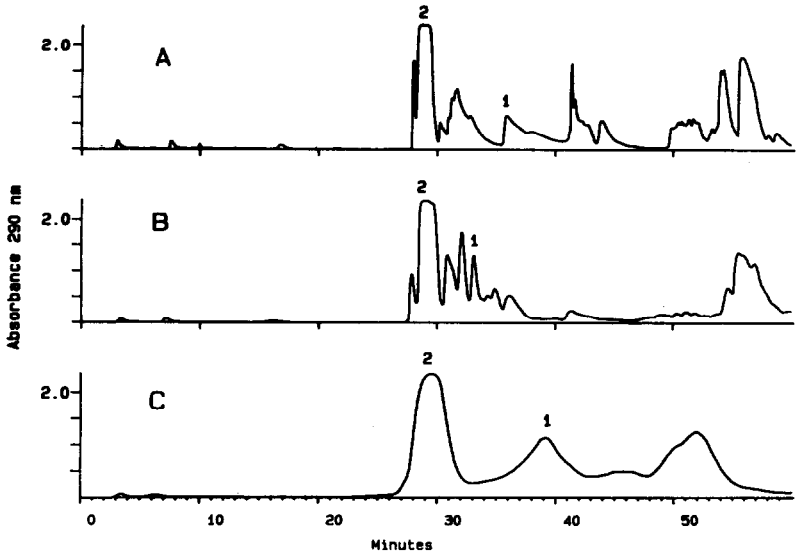

Fig. 2. Effect of silica particle size on the resolution of kadsurenone (1) and futoquinol (2). A 10-mg amount of extract per 150 μl methylene chloride was separated on 30 cm × 0.39 cm I.D. columns packed with 10-μm (A), 15-μm (B) and 37–55-μm (C) silica. Eluents, (A) hexane, (B) methylene chloride, (C) ethyl acetate and (D) acetonitrile, were combined as follows: 0–5 min A–B (25:75), 5–10 min linear gradient to 100% B, 10–15 min hold at 100% B, 15–35 min gradient to B–C–D (59:37:4), 35–45 min gradient to C–D (96:4) at a flow-rate of 1 ml/min. Detection, 290 nm, 2.0 a.u.f.s.

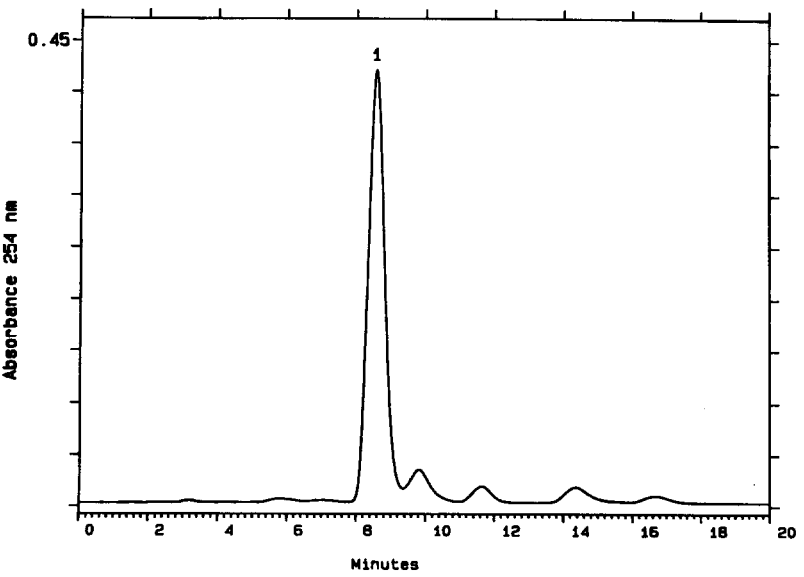

Fig. 3. RP-HPLC analysis of kadsurenone (1) in 20 μl of fraction 33, from the 15-μm silica separation, on a 15 cm × 0.39 cm I.D., 5-μm Delta Pak C_{18} column, using water–methanol (35:65) at a flow-rate of 0.5 ml/min. Detection, 254 nm, 0.45 a.u.f.s.

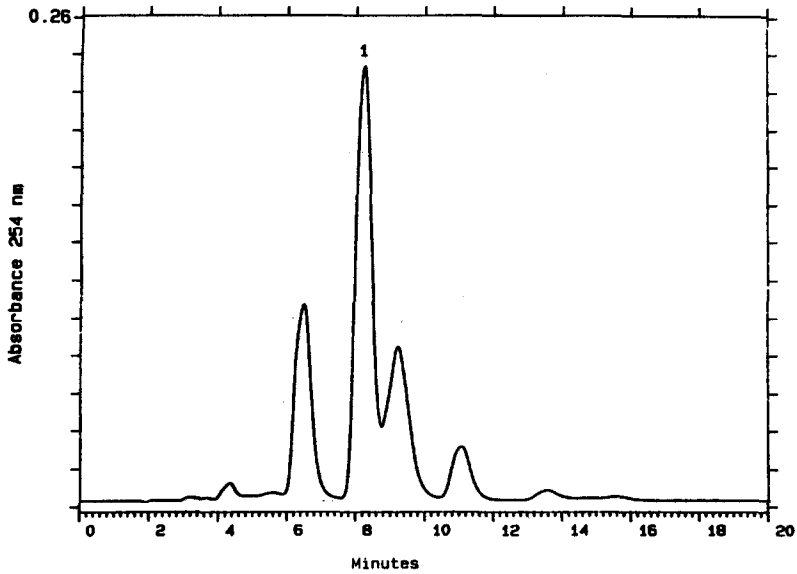

Fig. 4. RP-HPLC analysis of kadsurenone (1) in 20 μl of fraction 40 from the 37–55-μm silica separation. Conditions as in Fig. 3, 0.26 a.u.f.s.

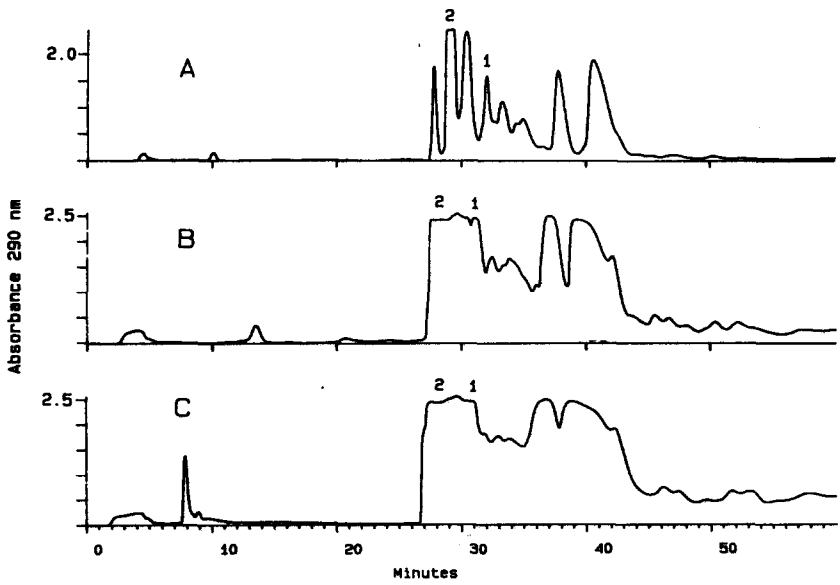

Fig. 5. Preparative-scale separation of 1 g/30 ml (A) 5 g/150 ml (B) and 6.5 g/200 ml (C) of crude extract in hexane–methylene chloride (25:75) on a 30 cm × 4.7 cm I.D. Prep Pak 15-μm silica cartridge. Eluents and conditions as in Fig. 2. Flow-rate was 80 ml/min. Detection was at 290 nm, 2.0 a.u.f.s. (A) and 2.5 a.u.f.s. (B and C). Peaks: 1 = kadsurenone; 2 = futoquinol.

Aliquots of crude extract dissolved in hexane–methylene chloride (25:75), containing 1, 5 and 6.5 g, were fractionated on a 30 cm × 4.7 cm I.D. Prep Pak cartridge packed with 15-μm silica. The chromatographic conditions were identical to those run on the 30 cm × 0.39 cm I.D. column, but at a flow-rate of 80 ml/min as shown in Fig. 5. Fractions (1 min) were collected, a 1-ml aliquot was reserved and the remainder dried and weighed. More than 80% of the total mass applied to the preparative column was recovered for each separation.

The 1-ml aliquots were dried and re-dissolved in methanol for analyses on the reversed-phase column. Fig. 6 shows the RP-HPLC of the futoquinol- and kadsurenone-containing fractions from the 6.5-g separation. The futoquinol elutes in fractions 28 (Fig. 6A) and 29 (Fig. 6B) with most of the kadsurenone in fraction 30 (Fig. 6C). The amount of kadsurenone (277 mg) present in fractions 30 and 31 from the 5- and 6.5-g separations was calculated by comparison of the peak areas to that of kadsurenone standard. The elution profiles of these fractions from the other normal-phase separations are very similar (results not shown).

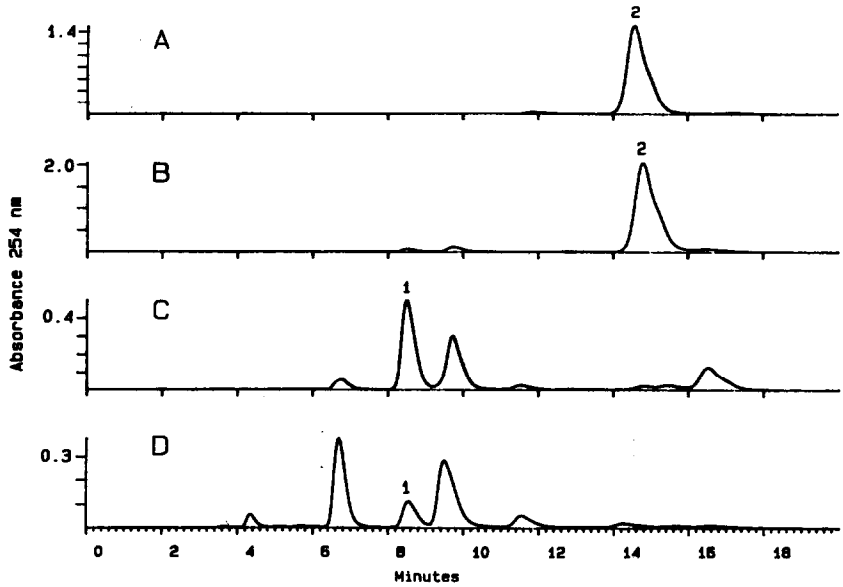

Fig. 6. RP-HPLC analyses of kadsurenone (1) and futoquinol (2) in 5 μl of 1-ml aliquots of fractions 29–32 (A–D) from the 6.5-g normal-phase separation. Conditions as in Fig. 3. Detection was at 254 nm, 1.0 a.u.f.s. (A), 1.4 a.u.f.s (B), 0.4 a.u.f.s. (C) and 0.15 a.u.f.s. (D).

Futoquinol (720 mg) was crystallized from these fractions using cold methanol. Fig. 7A shows the reversed-phase analysis of crystallized futoquinol and Fig. 7B shows the comparison of the UV spectrum of the isolated material to that of the standard.

Fractions 30 (1.2 g), containing kadsurenone, from the 5- and 6.5-g separations were dissolved in methanol, and a white crystalline precipitate, futoxide, was removed. The kadsurenone in the supernatant was then purified by reversed-phase HPLC on a 30 cm × 1.9 cm I.D., 15-μm Delta Pak C_{18} column using isocratic elution with

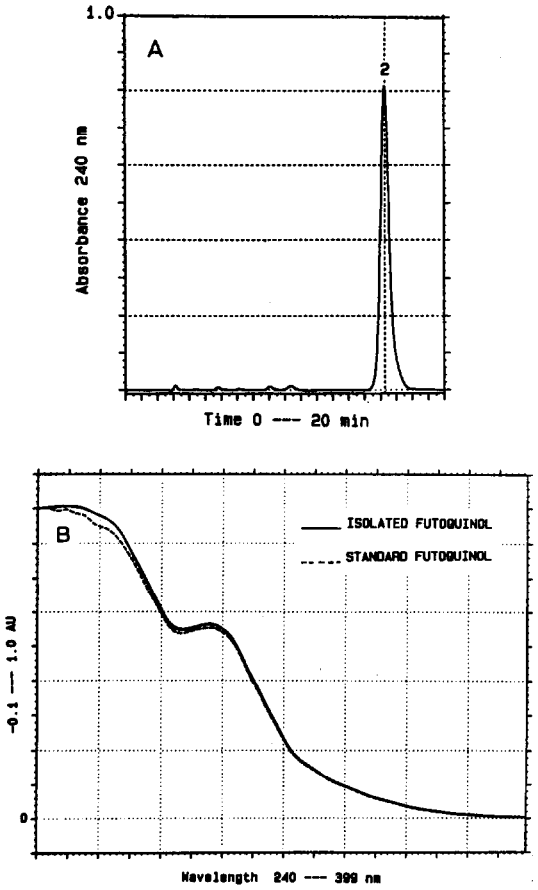

Fig. 7. RP-HPLC analysis of isolated futoquinol (2). Conditions as in Fig. 3. The chromatogram was monitored at 240 nm (A) and the spectra (B) of both the standard and isolated futoquinol scanned from 240 to 399 nm.

water–acetonitrile (55:45) at a flow-rate of 5 ml/min as shown in Fig. 8. Six injections of approximately 300 mg of supernatant were required to process all of the material. Kadsurenone has UV absorbance at 340 nm whereas the contaminants are best detected at lower wavelengths (290 nm). Dual-wavelength monitoring at 340 and 290 nm was used to distinguish the kadsurenone from contaminants facilitating the isolation of kadsurenone at overload conditions. Fractions 31 (0.6 g), containing kadsurenone, from the 5- and 6.5-g separations were similarly dissolved in methanol, and the futoxide was removed. The kadsurenone was purified on the 15-μm Delta Pak C_{18} column, but using water–methanol (40:60) as the mobile phase shown in Fig. 9. Six injections of approximately 70 mg of supernatant were required to process all the material. The water–methanol gave a better separation of the kadsurenone from the contaminants in this more complex fraction. A total of 266 mg of kadsurenone was recovered from these fractions, a 96% recovery. RP-HPLC analysis of this material is

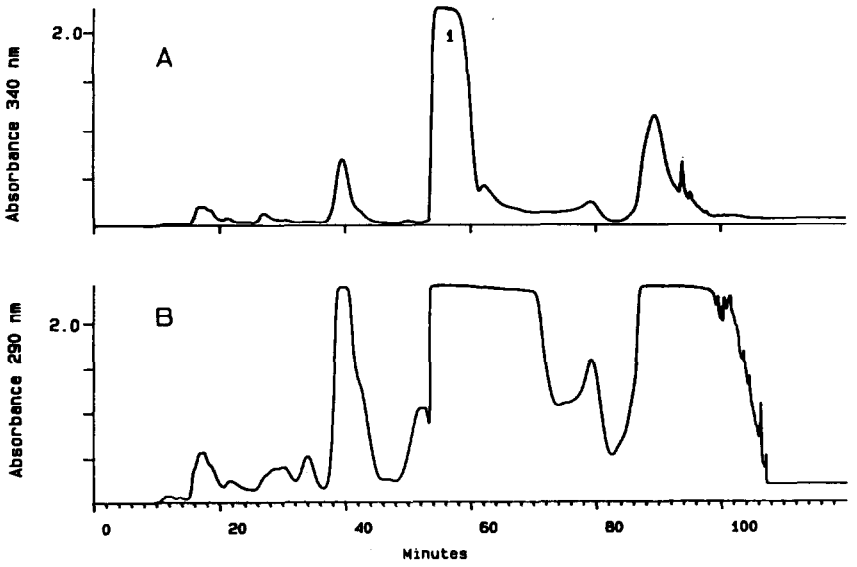

Fig. 8. Preparative RP-HPLC of kadsurenone (1) in 330 mg of fractions 30 from the 5- and 6.5-g normal-phase separation (Fig. 5C), on a 30 cm × 1.9 cm I.D., 15-μm Delta Pak C_{18} column. Isocratic elution at 5 ml/min with water–acetonitrile (55:45) was used. Detection was at 340 nm, 2.0 a.u.f.s. (A) and 290 nm, 2.0 a.u.f.s. (B).

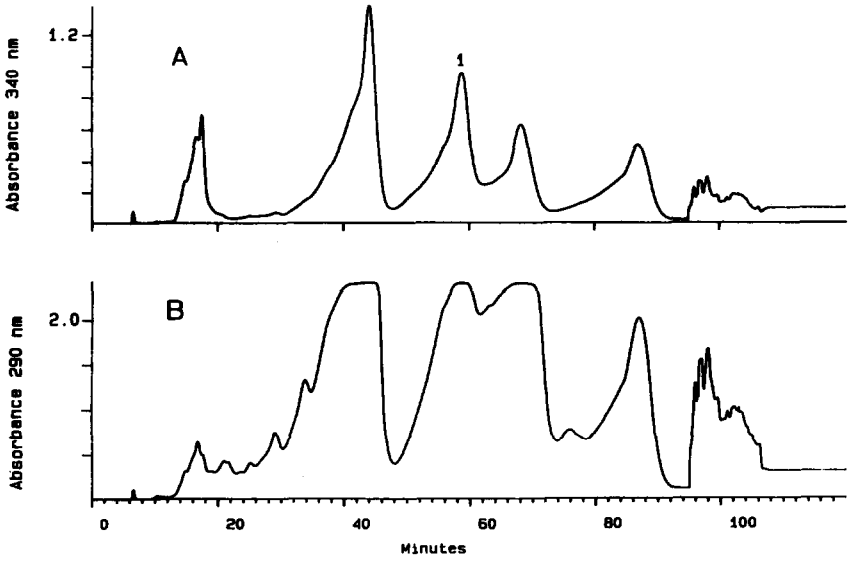

Fig. 9. Preparative RP-HPLC of kadsurenone (1) in 70 mg of fractions 31 from the 5- and 6.5-g normal-phase separation (Fig. 5B), on a 30 cm × 1.9 cm I.D., 15-μm Delta Pak C_{18} column. Isocratic elution at 5 ml/min with water–methanol (40:60) was used. Detection was at 340 nm, 1.2 a.u.f.s. (A) and 290 nm, 2.0 a.u.f.s. (B).

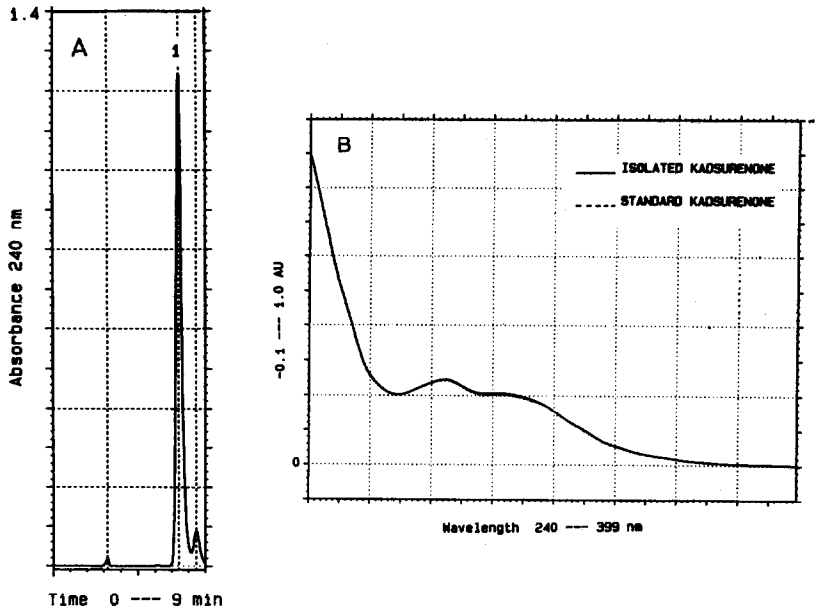

Fig. 10. RP-HPLC analysis of isolated kadsurenone (1). Conditions as in Fig. 3. The chromatogram was monitored at 240 nm (A) and the spectra (B) of both the standard and isolated kadsurenone scanned from 240 to 399 nm.

presented in Fig. 10A, while Fig. 10B shows the comparison of the UV spectra of the isolated kadsurenone to the standard.

In conclusion, the use of analytical HPLC simplified the task of methods development. The normal-phase separation of futoquinol and kadsurenone was developed on a 10-μm silica column by changing the selectivity of the mobile phase from hexane–ethyl acetate to methylene chloride–ethyl acetate. The introduction of two additional solvents, hexane and acetonitrile, into the gradient separation maximized both the amount of extract that was injected on to the column, as well as the resolution of kadsurenone from other plant constituents. Using this gradient, the resolution of the 10-, 15- and 37–55-μm columns was compared and the 15-μm silica selected for the preparative separation. The preparative normal-phase separation was carried out and futoquinol was crystallized from the fractions. Methods development on the reversed-phase column showed this technique useful, not only as an analytical technique for assessing the purity of fractions collected from the normal-phase separation, but as a preparative method for the final purification of kadsurenone.

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