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Note

Rapid separation and quantitative determination of khellin and visnagin in *Ammi visnaga* (L.) Lam. fruits by high-performance liquid chromatography

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Khellin (4,9-dimethoxy-7-methyl-5H-furo[3,2-*g*][1]benzopyran-5-one) and visnagin (4-methoxy-7-methyl-5H-furo[3,2-*g*][1]benzopyran-5-one), like other furochromones, are present in *Ammi visnaga* (L.) Lam. (Umbelliferae) fruits. Almost all furanochromones exhibit various biological activities[1–5]; khellin and visnagin, the most biologically active, are well known for their antispasmodic properties[5]. Khellin is used as a spamolitic agent in the therapy of asthma and angina pectoris and recently its use has been proposed for the treatment of vitiligo[6] and psoriasis[7]. Studies on the photogenic and mutagenic activity of visnagin have also been reported[8,9].

In the past the total chromones contained in *Ammi visnaga* fruits were extracted and separated by long, complicated procedures[10–13]; the concentrations of these compounds were determined by spectrophotometric[12] and gravimetric[14] methods.

Later, Chen *et al.*[15] studied the biosynthesis of khellin and visnagin of *Ammi visnaga* suspension cultures and reported the isolation and determination of visnagin by gas–liquid chromatography (GLC); the other furanochromones and furanocoumarins were separated by column and thin-layer chromatography and determined spectrophotometrically. The separation of khellin, visnagin and related compounds of aerial parts of *Ammi visnaga* has also been performed by gas chromatography[16]. Karawya *et al.*[17] could not obtain a sharp separation of khellin from visnagin in *Ammi visnaga* fruits by a thin-layer chromatographic method; the two furanochromones were determined by an improved spectrophotometric assay.

Recently, for the correct determination of its optimal therapeutic dose, khellin was evaluated in human serum and urine by a GLC assay[18] and a high-performance liquid chromatographic (HPLC) technique[19]. In pharmaceutical formulations khellin has been detected by GLC and determined by a fluorimetric method[20].

This paper describes a rapid and exhaustive extraction of khellin and visnagin from *Ammi visnaga* fruits and a specific, accurate and sensitive HPLC method for the separation and determination of the two furanochromones.

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EXPERIMENTAL

Apparatus and operating conditions

All chromatographic experiments were carried out using a Beckman Model 332 liquid chromatograph with a 20-μl sample loop, a Model 420 controller microprocessor, a Model 153 detector and a recorder. A 250 $\times$ 4.5 mm I.D. stainless-steel column packed with 5-μm Ultrasphere ODS (Altex, Berkeley, CA, U.S.A.) was employed for the chromatographic separation. The mobile phase was 50% methanol. The flow-rate was 1 ml/min, the chart speed was 0.5 cm/min, the detector sensitivity was 0.16 a.u.f.s., the pressure was 3000 p.s.i. and the absorbance was monitored at 254 nm. The injection volume was 20 μl in all experiments and the analyses were carried out at room temperature. For the spectrophotometric measurements a Beckman DU 8 spectrophotometer was used.

Materials

Methanol for HPLC was purchased from E. Merck (Darmstadt, F.R.G.) and acetic acid from J. T. Baker (Deventer, The Netherlands). Khellin and visnagin were kindly provided by Angelini (Ancona, Italy) and Inverni e Della Beffa (Milan, Italy), respectively. The water was deionized and filtered twice through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and finally degassed under vacuum before use. For sample filtration, 0.45-μm Millex-HV Millipore filters were used. All other reagents were commercial products of analytical-reagent grade.

Extraction of khellin and visnagin from plant material

Mature fruits were collected from *Ammi visnaga* plants, growing naturally in the "crete senesi", a large area with lower Pliocene clays south of Siena (Italy). The powdered *Ammi visnaga* fruits were weighed and homogenized in 10 volumes of acetic acid–water (1:1) with a Potter Elvehjem homogenizer. After centrifugation at 20,000 *g* for 20 min, the precipitate was further extracted with 5 volumes of acetic acid–water (1:1), homogenized and centrifuged at 20,000 *g* for 20 min. The two clear supernatants were collected, filtered and analysed by HPLC. The absence of furanochromones from the last precipitate was established by adding a few drops each of ethanol, an ethanolic solution of *m*-dinitrobenzene (2%) and potassium hydroxide solution (50%), as previously described[17]. The supernatant from a subsequent extraction was analysed by our HPLC method to detect possible traces of khellin and visnagin; in this experiment the detector sensitivity was 0.04 a.u.f.s.

Calibration graphs

To construct the calibration graphs, solutions of khellin and visnagin containing from 10 to 60 μg/ml of acetic acid–water (1:1) were prepared. The exact amount of each substance was determined by UV measurements. The millimolar absorption coefficient for khellin was 28 l mol^{-1} cm^{-1} at 250 nm and for visnagin 30 l mol^{-1} cm^{-1} at 245 nm[21]. These solutions were processed using the HPLC conditions described above. The data for the calibration graphs were subjected to the least-squares linear regression analysis and the resulting equations were utilized for the calculation of the concentrations of the two furanochromones in the plant material.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic separation of standard khellin and visnagin obtained in less than 30 min with k' values of 5.3 and 7.0, respectively. The mean recovery of known amounts of khellin injected into the chromatographic column was 97.9% (S.D. = 4.6%; C.V. = 4.7%) in six different experiments. Similar experiments were performed for visnagin, with a mean recovery of 97.6% (S.D. = 4.3%; C.V. = 4.4%). The calibration graphs for khellin and visnagin were constructed using the calibration equation reported in Table I.

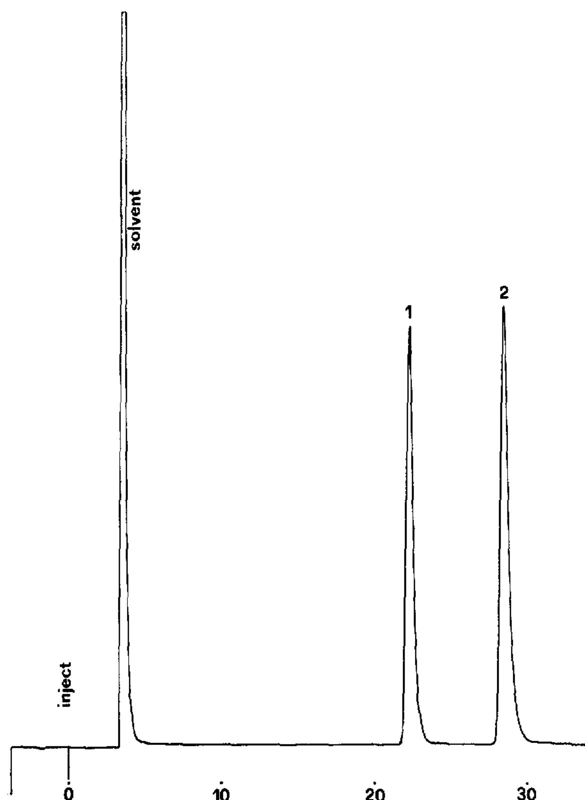

Fig. 1. HPLC separation of khellin and visnagin standards. 1 = Khellin; 2 = visnagin. Ordinate: absorbance (254 nm); abscissa: retention time (min). Operating conditions as described under Experimental.

The amount of the two furanochromones injected into the chromatographic column was varied between 0.2 and 1.2 μg. Each point was the average of at least five different injections. The linearity and fit for all points were very good.

The method was applied to plant extracts obtained from *Ammi visnaga* fruits. The extraction of khellin and visnagin was achieved by simple homogenization instead of the extremely time-consuming procedures used in the past. The complete removal of furanochromones from the plant material was accurately assayed as described under Experimental. No violet colour was obtained in the last precipitate and

TABLE I

CALIBRATION EQUATIONS FOR THE HPLC DETERMINATION OF KHELLIN AND VISNAGIN

Compound	*Equation**	*r*
Khellin	$y = 14.7x - 0.2$	0.995
Visnagin	$y = 18.37x - 0.011$	0.997

* y = peak height; x = weight of the sample.

no traces of khellin and visnagin were detectable in the supernatant from a third extraction.

In Fig. 2, the typical chromatogram of an extract of *Ammi visnaga* fruits shows the excellent separation of khellin from visnagin and of the two drugs from other unknown compounds. The identities of the two furanochromones peaks were established by the retention time and by the addition of standard compounds to the extract. The retention times of khellin and visnagin from plant extract were identical with those of added standards. The peak shape was symmetrical and characteristic of khellin and visnagin standard peaks.

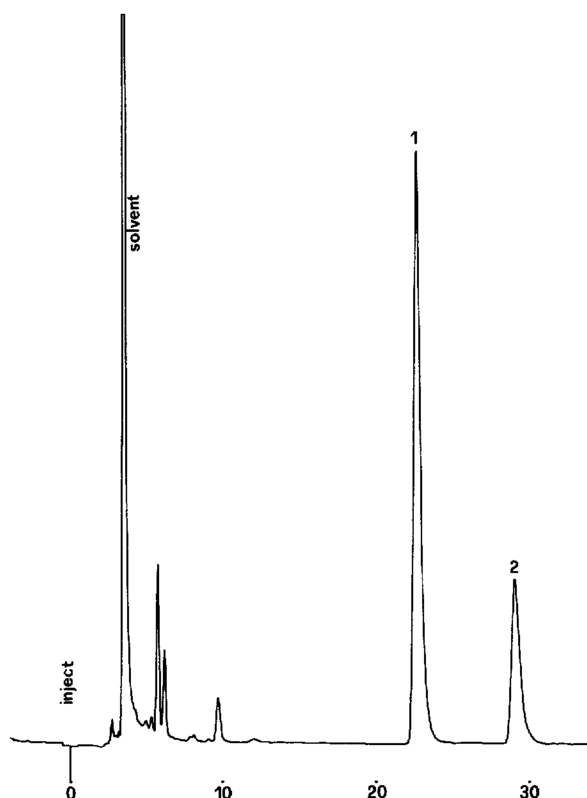

Fig. 2. Typical HPLC trace of plant extract. 1 = Khellin; 2 = visnagin. Ordinate: absorbance (254 nm); abscissa: retention time (min). The plant extract was diluted 20-fold; operating conditions as described under Experimental.

TABLE II

REPRODUCIBILITY AND ACCURACY OF THE DETERMINATION OF KHELLIN AND VISNAGIN IN SPIKED PLANT EXTRACT

Compound	*Amount added (μg/ml)*	*Amount found (μg/ml) (mean ± S.D., n = 8)*	*C.V. (%)*
Khellin	15	15.1 ± 0.6	3.9
	20	19.9 ± 0.3	1.5
	25	25.9 ± 0.5	1.9
	30	31.2 ± 0.5	1.6
Visnagin	15	14.9 ± 0.5	3.3
	20	20.2 ± 0.9	2.2
	25	25.8 ± 0.4	1.5
	30	30.7 ± 0.8	1.6

Reproducibility and accuracy studies were performed at four different concentrations of khellin and visnagin in spiked control plant extracts. The coefficient of variation was about 2.1%. The results are given in Table II.

The determination of the two chromones in *Ammi visnaga* fruits was carried out using the calibration equations and Table III reports the amounts of khellin and visnagin in *Ammi visnaga* fruit extracts obtained with five different extractions. The mean contents of khellin and visnagin were 1.17% (S.D. = 0.08%; C.V. = 6.8%) and 0.33% (S.D. = 0.03%; C.V. = 9%), respectively.

TABLE III

AMOUNT OF KHELLIN AND VISNAGIN IN *AMMI VISNAGA* FRUITS

The values are expressed as mg per 100 mg tissue. Each value represents the average of at least three assays.

Khellin (%)	*Visnagin (%)*
1.11	0.31
1.09	0.35
1.21	0.29
1.29	0.32
1.14	0.36

We believe that the method described will be very useful for evaluating the amounts of khellin and visnagin in plant materials and for clarifying the metabolic pathways that have already been proposed and which are still obscure[15,16,22].

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