ELSEVIER

Journal of Chromatography A, 797 (1998) 305–309

JOURNAL OF CHROMATOGRAPHY A

Determination of furanochromones and pyranocoumarins in drugs and *Ammi visnaga* fruits by combined solid-phase extraction–high-performance liquid chromatography and thin-layer chromatography–high-performance liquid chromatography

Grażyna Zgórka*, Teresa Dragan, Kazimierz Głowniak, Edyta Basiura

Department of Pharmacognosy, University Medical Academy, Peowiaków 12 St., 20-007 Lublin, Poland

Abstract

A new, simple and rapid solid-phase extraction method for the determination of furanochromones and pyranocoumarins in *Ammi visnaga* L. fruits and pharmaceuticals by reversed-phase high-performance liquid chromatography (RP-HPLC) was developed. The isolation of compounds examined was carried out on octadecyl BakerBond SPE columns using various concentrations of methanol, acetonitrile and tetrahydrofuran in water. High and reproducible recoveries were obtained. To compare the results of quantitative analysis a preparative TLC procedure was also elaborated and carried out. © 1998 Elsevier Science B.V.

Keywords: *Ammi visnaga*; Fruits; Pharmaceutical analysis; Furanochromones; Pyranocoumarins; Khellin; Visnagin; Visnadin

1. Introduction

Khellin, visnagin and visnadin are the main principles of *Ammi visnaga* fruits. They are respectively, derivatives of furanochromones (khellin, visnagin) and pyranocoumarins (visnadin).

As plant active constituents, showing calcium channel blocking activity [1,2], they are the components of pharmaceutical preparations applied in the treatment of coronary diseases and bronchial asthma [3]. In the literature, methods describing isolation and quantitative analysis of furanochromones are mainly time consuming procedures comprising liquid–solid and liquid–liquid extraction or thin-layer chromatography (TLC) separation followed by colorimetric and spectrophotometric [4–7], polarographic [8] or fluorometric [9,10] determinations. Numerous high-performance liquid chromatography (HPLC) methods for the determination of furanochromones (especially khellin) have been also reported [11–14]. However, they were often characterized by a long elution time or required complex sample preparation procedures prior to the exact HPLC analysis, hence lacked the accuracy of quantitative results.

There are also no data for quantitative analysis of pharmacologically active pyranocoumarins in *A. visnaga* fruits.

In contrast, solid-phase extraction (SPE), in this report described, seems to be a very effective, fast and reproducible method for preconcentration, purification and, in combination with HPLC, quantitation

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01173-4

of furanochromones and pyranocoumarins, not only in plant alcoholic extracts but also in complex pharmaceuticals, derived from *A. visnaga* fruits. To prove it, two combined SPE–HPLC and TLC–HPLC procedures were elaborated and compared.

2. Experimental

2.1. Apparatus

HPLC analysis was performed using a Hewlett-Packard (Palo Alto, CA, USA) Model 1050 liquid chromatograph equipped with a 20-μl sample injector (Rheodyne, Cotati, CA, USA) and a variable-wavelength UV–Vis detector. The chromatograms were recorded at 320 nm with a 3396A reporting integrator (Hewlett-Packard). A stainless steel column (200×4.6 mm I.D.) packed with 5 μm ODS Hypersil (Shandon, Cheshire, UK) was used.

2.2. Analytical method

Separation of khellin, visnagin and visnadin was accomplished by isocratic elution. Two mobile phases: acetonitrile–water (45:55, v/v) – for the quantitation of furanochromones in complex pharmaceutical formulations (Kelastmin, Kelicardina) – and (60:40, v/v) were used, at a flow-rate of 1 ml min^{-1} and at an ambient temperature.

The quantitative results of compounds examined were calculated using calibration curves.

2.3. Reagents and materials

Standards of khellin, visnagin and visnadin were purchased from Carl Roth (Karlsruhe, Germany). One mg of each compound was dissolved in methanol (10 ml) and different dilutions (0.01–0.1 mg ml^{-1}) were next prepared and applied to the column. Samples of 10 μl were injected.

All other reagents [methanol (MeOH), acetonitrile (MeCN), tetahydrofuran (THF)] were of chromatographic grade (Merck, Darmstadt, Germany) and in all experiments bidistilled water was used.

For the SPE procedure, an octadecyl BakerBond column (500 mg, 3 ml), supplied by J.T. Baker (Phillipsburg, NJ, USA) was used. Extraction was performed on a 12-port vacuum manifold processor (system Baker SPE-12G).

A. visnaga fruits were purchased from Herbapol (Kraków, Poland). Commercially available, liquid pharmaceutical preparations: Tinctura Ammi visnagae (Herbapol, Kraków, Poland), Kelastmin and Kelicardina (Herbapol, Wrocław, Poland) were examined.

2.4. Sample preparation

Dry, powdered *A. visnaga* fruits (1 g) were refluxed with methanol (10 ml) on a water bath for 30 min. After cooling the liquid was carefully decanted and the plant material was reextracted with another portion of methanol (5 ml) for 15 min. Both supernatants were combined, filtered and submitted to SPE and TLC isolation procedures.

2.5. SPE method

Methanolic (20%) extracts of *A. visnaga* fruits or, diluted to the same percentage, liquid pharmaceutical formulations were loaded onto octadecyl SPE microcolumns activated previously with methanol (10 ml), followed by bidistilled water (10 ml). The support was then washed with water (1 ml) to remove matrix and vacuum-dried for 1 min.

The next step was the selective elution of compounds examined. The cartridges were washed with 10 ml of 60% (v/v) methanol (series I), 40% (v/v) acetonitrile (series II) and 30% (v/v) tetrahydrofuran (series III), respectively, to remove fractions of furanochromones and next with 10 ml of 80% (v/v) methanol (series Ia), 70% (v/v) acetonitrile (series IIa), 60% (v/v) tetrahydrofuran (series IIIa) to elute pyranocoumarins (visnadin). The eluting solvents were passed through the sorbent beds at a flow-rate of 0.5 ml min^{-1}. All collected eluates were directly analysed by HPLC.

2.6. TLC preparative method

Parallel samples of methanolic extracts from *A. visnaga* fruits or liquid pharmaceuticals were spotted linearly on TLC plates. Preparative chromatography was performed on 20×10 cm glass plates covered with a 0.5-mm layer of Kieselgel 60 G (Merck) by

means of a coating apparatus (Quickfit, UK) and activated before use at 110°C. Chromatograms were run with ethyl acetate over a distance of 9 cm in horizontal DS chambers (Chromdes, Lublin, Poland). The examination of bands under UV light (λ=366 nm) showed visnagin as pale-yellow at R_F=0.51, khellin as yellowish–brown at R_F=0.57 and visnadin as violet at R_F=0.89.

The located common band of furanochromones and the second one of visnadin were separately scraped off and the compounds eluted from silica with methanol (3×10 ml). After centrifuging, supernatants were combined, partially evaporated under vacuum, filtered and finally analysed by HPLC.

2.7. Recovery tests

For both (SPE and TLC) methods recovery tests were performed. A standard solution of khellin, visnagin and visnadin (c=1 mg ml^{-1}) in 20% (v/v) methanol was prepared. Five ml volumes of this aliquot were either directly submitted to previously described SPE procedure or, combined with methanolic (20%) extracts of *A. visnaga* fruits (5 ml) and passed through octadecyl cartridges as samples fortified.

A similar procedure, using the same concentration of standards in 100% methanol, was repeated for the TLC method. 0.5-ml volumes of the standard solution or spiked methanolic extracts of *A. visnaga* were spotted linearly on TLC plates and then developed and analysed using previously described operating conditions.

Table 1
Mean recoveries (%±R.S.D, %; n=5) of khellin, visnagin and visnadin obtained for different eluents on octadecyl columns for pure standards (A) and fortified samples (B)

Compound	Mean recovery (%)±R.S.D (%); n=5	
	A	B
Khellin	101.3±0.6[a]	102.8±3.3[a]
	102.3±1.0[b]	99.9±0.3[b]
	100.6±3.0[c]	100.5±3.0[c]
Visnagin	102.0±0.8[a]	103.2±2.7[a]
	102.0±2.6[b]	96.9±1.4[b]
	96.0±5.0[c]	98.3±1.5[c]
Visnadin	102.4±4.1[d]	104.9±2.4[d]
	99.9±5.8[e]	102.4±6.0[e]
	101.1±4.0[f]	97.7±4.7[f]

SPE eluents: [a] 60% MeOH; [b] 40% MeCN; [c] 30% THF; [d] 80% MeOH; [e] 70% MeCN; [f] 60% THF.

Table 2
Mean recoveries (%±R.S.D, %; n=5) of khellin, visnagin and visnadin obtained by the application of combined TLC–HPLC methods for pure standards (A) and fortified samples (B)

Compound	Mean recovery (%)±R.S.D (%); n=5	
	A	B
Khellin	87.3±7.4	84.3±7.3
Visnagin	84.1±4.6	84.7±8.1
Visnadin	81.6±3.1	85.9±6.7

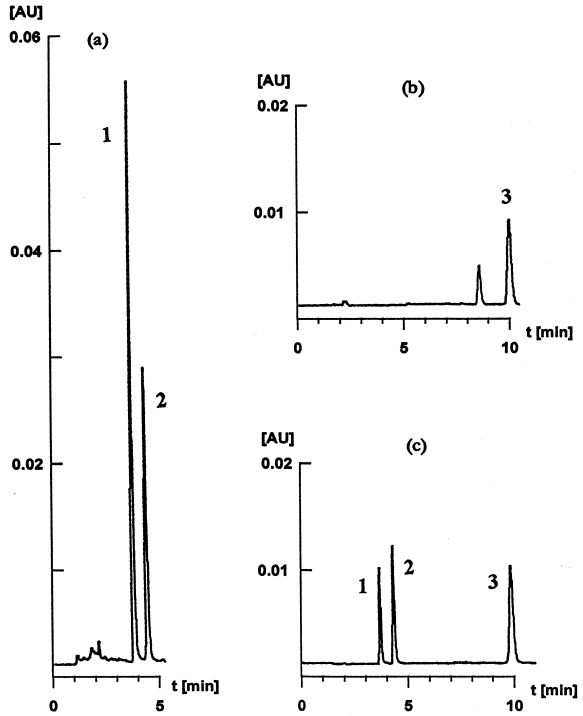

Fig. 1. Separation of khellin (1), visnagin (2) and visnadin (3) in SPE-eluates: (a) and (b), obtained from *A. visnaga* fruits, carried out on a ODS Hypersil (200×4.6 mm I.D., 5 μm) column under isocratic conditions. Mobile phase: acetonitrile–water (60:40, v/v). Flow-rate, 1 ml min^{-1}; detection, 320 nm; (c) the standards of (1), (2) and (3).

3. Results and discussion

Sample preparation and extraction of compounds from biological matrices seem to be the most important stages for further successful quantitative analysis. The present investigations were undertaken in order to develop SPE as a sample preparation technique, capable of easy purifying, preconcentrating and, in combination with HPLC, quantitating of furanochromones and pyranocoumarins in *A. visnaga* fruits and the pharmaceuticals originating from this plant material. Additionally TLC preparative procedure was elaborated. The comparison of both methods showed that SPE enabled very high recoveries of compounds examined with a good reproducibility of quantitative results, for both pure standards and samples fortified. The mean recoveries of khellin were 99.9–102.8% with R.S.D. of 0.3–3.3%; for visnagin 96.0–103.2% with R.S.D. of 0.8–5.0% and for visnadin 97.7–104.9% with R.S.D. of 2.4–6.0% (Table 1).

The described TLC preparative method is also simple and convenient as well as guarantees the good separation of compounds examined but it does not provide satisfying recoveries (~85% for all compounds) and reproducibility (higher values of R.S.D.) – see Table 2.

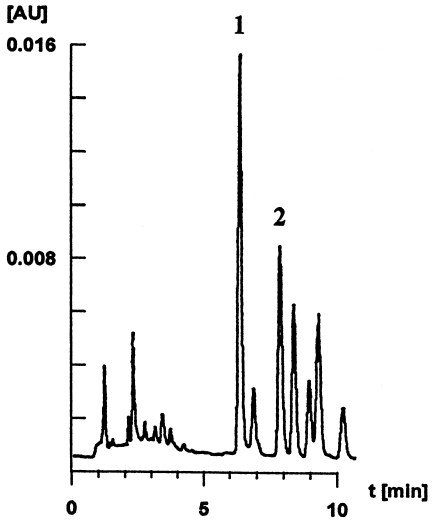

Fig. 2. Separation of khellin (1) and visnagin (2) in 60% (v/v) methanolic SPE-eluate, obtained from a complex pharmaceutical formulation: Kelastmin, carried out on a ODS Hypersil (200×4.6 mm I.D., 5 μm) column under isocratic conditions. Mobile phase: acetonitrile–water (45:55, v/v); flow-rate and detection: see Fig. 1.

Table 3
Comparison of the content of furanochromones and pyranocoumarins in *A. visnaga* fruits obtained by the application of two combined SPE–HPLC and TLC–HPLC methods

Compound	Mean amount (% dry mass±R.S.D, %; $n=5$)	
	SPE–HPLC	TLC–HPLC
Khellin	2.89±0.09[a] 2.91±0.15[b] 3.03±0.04[c]	2.27±0.13
Visnagin	1.16±0.03[a] 1.17±0.07[b] 1.28±0.06[c]	0.93±0.06
Visnadin	0.45±0.01[d] 0.46±0.02[e] 0.45±0.03[f]	0.37±0.02

SPE eluents: [a] 60% MeOH; [b] 40% MeCN; [c] 30% THF; [d] 80% MeOH; [e] 70% MeCN; [f] 60% THF.

As regards HPLC analysis – Figs. 1 and 2 present the separation of khellin, visnagin and visnadin in eluates obtained by SPE method. The reversed-phase system used gave very well resolved and symmetrical peaks at very short times of analysis (below 10 min). Careful equilibrium with the isocratic mobile

Table 4
Comparison of the content of furanochromones and pyranocoumarins, in the drug: Tinctura *Ammi visnagae*, obtained by the application of two combined SPE–HPLC and TLC–HPLC methods

Compound	Mean amount (mg ml^{-1}±S.D.; $n=5$)	
	SPE–HPLC	TLC–HPLC
Khellin	0.76±0.01[a] 0.74±0.01[b] 0.76±0.02[c]	0.67±0.13
Visnagin	0.32±0.01[a] 0.31±0.01[b] 0.32±0.01[c]	0.29±0.06
Visnadin	0.15±0.00[d] 0.15±0.00[e] 0.15±0.00[f]	0.11±0.02

SPE eluents: [a] 60% MeOH; [b] 40% MeCN; [c] 30% THF; [d] 80% MeOH; [e] 70% MeCN; [f] 60% THF.

Table 5
Comparison of the content of furanochromones and pyranocoumarins, in the drug: Kelicardina, obtained by the application of two combined SPE–HPLC and TLC–HPLC methods

Compound	Mean amount (mg ml^{-1} ± S.D.; n=5)	
	SPE–HPLC	TLC–HPLC
Khellin	2.05±0.11[a]	1.43±0.08
	2.12±0.08[b]	
	2.13±0.08[c]	
Visnagin	0.81±0.05[a]	0.57±0.02
	0.83±0.03[b]	
	0.84±0.03[c]	
Visnadin	0.28±0.02[d]	0.19±0.02
	0.30±0.01[e]	
	0.29±0.02[f]	

SPE eluents: [a] 60% MeOH; [b] 40% MeCN; [c] 30% THF; [d] 80% MeOH; [e] 70% MeCN; [f] 60% THF.

phases was also essential for run-to-run reproducibility. The HPLC method used seemed to be sensitive – the detection limit was about 0.1 ng for all compounds at a signal-to-noise ratio of 3:1 for the peak heights. It is also the first time that visnadin was quantitatively analysed in *A. visnaga* fruits and pharmaceuticals.

Table 6
Comparison of the content of furanochromones and pyranocoumarins, in the drug: Kelastmin, obtained by the application of two combined SPE–HPLC and TLC–HPLC methods

Compound	Mean amount (mg ml^{-1} ± S.D.; n=5)	
	SPE–HPLC	TLC–HPLC
Khellin	0.33±0.01[a]	0.27±0.02
	0.32±0.01[b]	
	0.32±0.01[c]	
Visnagin	0.15±0.00[a]	0.11±0.02
	0.15±0.01[b]	
	0.14±0.01[c]	
Visnadin	0.05±0.00[d]	0.04±0.01
	0.05±0.00[e]	
	0.05±0.00[f]	

SPE eluents: [a] 60% MeOH; [b] 40% MeCN; [c] 30% THF; [d] 80% MeOH; [e] 70% MeCN; [f] 60% THF.

In conclusion, combined SPE–HPLC and TLC–HPLC methods, presented in this paper, are easy, rapid and reproducible procedures. However, regarding the precision of quantitative results (Tables 1–6), the former seems to be more useful for the quantitation of khellin, visnagin and visnadin. As a simple and sensitive method, SPE–HPLC can be recommended for simultaneous, routine studies on the content of furanochromones and pyranocoumarins in the plant material and pharmaceutical preparations.

References

[1] J. Duarte, F. Perez-Vizcaino, A.I. Torres, A. Zarzuelo, J. Jimenez, J. Tamargo, Eur. J. Pharmacol. 286 (1995) 115.
[2] H.W. Rauwald, O. Brehm, K.P. Odenthal, Planta Med. 60 (1994) 101.
[3] W.C. Evans (Ed.), Pharmacognosy, University Press, Cambridge, 1989, Ch. 6, p. 422.
[4] S.I. Balbaa, A.Y. Zaki, S.M. Abdel-Wahab, Planta Med. 16 (1968) 329.
[5] M.S. Karaway, G. Nour, A. Sina, Planta Med. 19 (1971) 368.
[6] M.S. Karaway, M.A. El-Keiy, A. Sina, G. Nour, J. Pharm. Sci. 59 (1970) 1025.
[7] M.S. Karaway, A.N. Girgis, S.E. Khayyal, J. Drug Res. 3 (1971) 207.
[8] N.P. Dziyba, Y.E. Orlov, L.Y. Sirenko, R.V. Dorofeeva, Herba Pol. 7 (1971) 72.
[9] S.H. Hilal, A.S. Radwan, M.Y. Haggag, F.R. Melek, O.D. El-Gindi, Egypt. J. Pharm. Sci. 23 (1982) 203.
[10] M.A. Abdel-Salam, M.E. Abdel-Hamid, Z.F. Mahmoud, Anal. Lett. 18B1 (1985) 35.
[11] P. Martelli, L. Bovalini, S. Ferri, G.G. Franchi, J. Chromatogr. 301 (1984) 297.
[12] F.K. El-Fiky, R.P. Remmel, E.J. Staba, Planta Med. 55 (1989) 446.
[13] M.A. Abounassif, E.A. Gad-Kariem, A.M. Wahbi, Il Farmaco 45 (1990) 465.
[14] M.M. El-Domiaty, J. Pharm. Sci. 81 (1992) 475.