Journal of Chromatography, 614 (1993) 324–327
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6754

Short Communication

Determination of khellin in serum by gas chromatography

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(First received April 4th, 1992; revised manuscript received January 13th, 1993)

ABSTRACT

A rapid gas chromatographic assay has been developed for the separation and determination of khellin in human serum. Using a DB-17 capillary column and a simple chloroform extraction, khellin and an internal standard, trioxsalen, were separated from endogenous substances without further clean-up. Spiked serum samples in the range 0.11–1.1 μg/ml were assayed and a linear calibration curve obtained.

INTRODUCTION

Khellin (Fig. 1) is a furanochromone present in the plant *Ammi visnaga* (L.) Lam. (Umbelliferae) [1,2]. It is active as a vasodilator and is also used for the treatment of vitiligo, a condition in which the skin is disfigured by loss of melanin pigment [1–3]. The availability of a sensitive analytical method is important because of khellin's narrow therapeutic index. El-Yazigi and Said [4] achieved a limit of quantitation of 0.10 μg/ml using reversed-phase column LC and UV detection. Using GC on packed columns, Said and Babikar [5] achieved a limit of 1 μg/ml. Martelli *et al.* [1] also quantitated khellin using HPLC but their method was used to assay khellin in the *Ammi visnaga* plant rather than in serum. By employing capillary GC, trioxsalen (Fig. 1) as an internal

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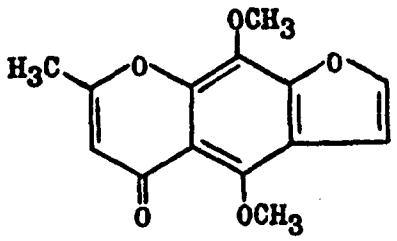
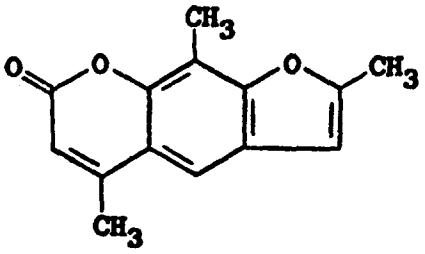
0378-4347/93/$06.00



KHELLIN



TRIOXSALEN

Fig. 1. Structures of khellin and trioxsalen.

standard and flame-ionization detection, we were able to achieve a ten-fold increase in sensitivity over previously reported GC methods.

EXPERIMENTAL

Reagents and supplies

Khellin was obtained from Sigma (St. Louis, MO, USA), trioxsalen from Aldrich (Milwaukee, WI, USA), chloroform from EM Science (Gibbstown, NJ, USA), sodium bicarbonate from J.T. Baker (Phillipsburg, NJ, USA) and sodium hydroxide and sodium sulfate from Mallinckrodt (Paris, KY, USA). All chemicals and solvents were reagent grade.

Equipment

A Hewlett-Packard Model 5890 gas chromatograph equipped with a flame-ionization detector and integrator was used. The column was a DB-17 capillary, 15 m × 0.32 mm I.D. with a 0.25-μm-thick film (J&W Scientific, Folsom, CA, USA). The extractions were carried out using a Thermolyne Speci-Mix Model M-26125 and the extracts concentrated in 1.0-ml tapered vials (Alltech Assoc., Deerfield, IL, USA).

Chromatographic conditions

Helium was used as carrier gas at 0.104 MPa head pressure and also as make-up gas; splitless injection with a sampling time of 0.5 min; injector temperature, 275°C; detector temperature, 300°C. Oven temperature program: start 45°C, hold 0.5 min, then ramp 30°C/min to 240°C and hold.

Direct standards in chloroform

Stock solutions of khellin (0.27 mg/ml) and trioxsalen (0.45 mg/ml) were prepared in chloroform. Aliquots of the khellin solution and 50 μl of the trioxsalen solution were then added to 50-ml volumetric flasks and diluted to volume with chloroform to give working standards containing 0.11, 0.22, 0.44, 0.87 and 1.1 μg/ml khellin with trioxsalen constant in all solutions at 0.45 μg/ml.

Preparation of spiked human serum samples

A khellin spiking solution was prepared by diluting 1.0 ml of the khellin stock solution to 50.0 ml with chloroform. Aliquots of this solution (10, 20, 40, 80 and 100 μl) were added to small glass tubes (1 cm × 10 cm). Four tubes were prepared at each of the concentration levels. The aliquots were allowed to evaporate spontaneously to dryness at ambient temperature. A 0.5-ml volume of human serum was then added to each tube, and the tubes were vortex-mixed briefly to dissolve the khellin residue in the serum. This yields spiked serum samples containing khellin at concentrations of 0.11, 0.22, 0.44, 0.87 and 1.1 μg/ml, the same levels as the working standards previously prepared.

Standard extraction curve

A 0.5-ml volume of a 0.5 *M* pH 10 bicarbonate buffer was added to the 0.5 ml of spiked serum followed by 0.5 ml of water-saturated chloroform containing the internal standard, trioxsalen, at a concentration of 0.45 μg/ml. The tubes were rocked on a Thermolyne Speci-Mix for 15 min and then centrifuged at 2000 *g* for 15 min. The

aqueous phase was drawn off and discarded using a disposable pipet and the organic extract poured off into a 1.0-ml tapered vial. Using this technique, it was possible to separate the organic extract completely from the aqueous phase. The extract was then concentrated slowly to approximately 25 μl with a gentle stream of nitrogen in a 40°C water bath. Approximately 25 mg of anhydrous sodium sulfate were added to the vial and the contents vortex-mixed briefly. A 0.5-μl injection was then made into the gas chromatograph.

RESULTS AND DISCUSSION

Other workers [4,5] have used theophylline or hyoscine as an internal standard. These compounds, however, are not structually related to khellin. We chose trioxsalen, a compound structurally related to khellin, for our internal standard. We buffered our samples at basic pH prior to extraction after observing that the chloroform extracts were cleaner than those made at neutral pH. Although trioxsalen is a lactone and might be subject to loss by hydrolysis at basic pH, we did not observe such loss at pH 10 under the experimental conditions. Neither khellin nor trioxsalen has ionizable protons, so their partition between chloroform and water is not a function of pH. At pH 10, khellin and trioxsalen were separated from endogenous substances without any further clean-up of the chloroform extracts. The retention times of khellin and trioxsalen were 8.1 and 7.7 min, respectively. Validations were run on two separate days using newly prepared standards and spiked serum on each day. Results are presented in Table I. Chromatograms of blank serum and spiked serum extract are presented in Fig. 2.

The calibration curve produced from spiked serum was linear from 0.11 to 1.1 μg/ml with a correlation coefficient of 0.988. The lower limit of quantitation was 0.11 μg/ml which was twice the noise level. Back-extraction of standard solutions of khellin and trioxsalen in chloroform with pH 10 buffer did not cause a significant change in the peak-area ratio of khellin to trioxsalen indicating 100% relative partitioning of the compounds between the two phases. Absolute extraction efficiency, calculated by dividing the slope of the

TABLE I

VALIDATION OF THE GAS CHROMATOGRAPHIC ANALYSIS

Actual concentration (μg/ml)	Observed concentration (mean ± S.D.) (μg/ml)	*n*	C.V. (%)	Mean error (%)
Day 1				
0.11	0.13 ± 0.010	4	4.0	18
0.21	0.25 ± 0.035	3	8.0	19
0.41	0.37 ± 0.036	3	5.5	9.8
0.83	0.73 ± 0.064	4	5.0	12
1.04	0.94 ± 0.11	4	6.6	6.3
Day 2				
0.11	0.13 ± 0.027	4	12.9	18
0.22	0.25 ± 0.015	4	4.0	13
0.44	0.42 ± 0.017	4	2.6	4.5
0.87	0.82 ± 0.10	4	9.8	6.7
1.10	0.93 ± 0.049	4	3.5	7.5

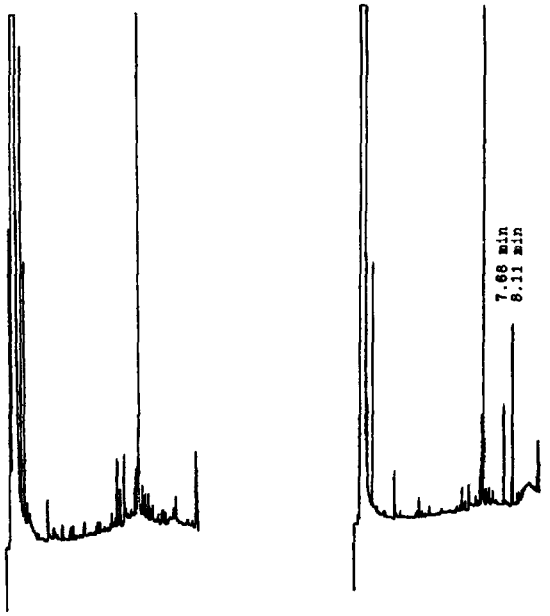


Fig. 2. Chromatograms of (left) blank human serum and (right) human serum spiked with khellin at a concentration of 1.1 μg/ml. Peak identification according to retention time: 7.68 min, trioxsalen (internal standard); 8.11 min, khellin.

spiked serum extraction curve by the slope of the curve of standards injected directly, averaged 84%.

REFERENCES

1 P. Martelli, L. Bovalini, S. Ferri and G. G. Franchi, *J. Chromatogr.*, 301 (1984) 297.
2 P. G. Harrison, B. K. Bailey and W. Steck, *Can. J. Biochem.*, 49 (1971) 964.
3 B. Ortel, A. Tenew and H. Honigsmann, *Curr. Probl. Dermatol.*, 15 (1986) 265.
4 A. El-Yazigi and S. A. Said, *J. Pharm. Sci.*, 69 (1980) 1434.
5 S. A. Said and K. H. Babikar, *Chromatographia*, 14 (1981) 425.