Fluorometric Determination of Khellin in Human Urine and Serum by High-Performance Liquid Chromatography Using Postcolumn Photoirradiation

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For the determination of khellin in urine and serum, fluorometry using HPLC-postcolumn photoirradiation has been developed. Khellin and visnagin of similar structure were separated on a column of Capcell Pak C₈. The mobile phase consisted of 40% (v/v) ethanol containing 75 mmol l⁻¹ H₂O₂. The postcolumn reagent, 70 mmol l⁻¹ KH₂PO₄-NaOH buffer (pH 12.7) containing 50% (v/v) ethanol, were mixed with the mobile phase, which was irradiated with ultraviolet light to induce fluorescence. The fluorescence was monitored with excitation at 378 nm and emission at 480 nm. The calibration graph for khellin was linear over the range of 65 – 2620 ng ml⁻¹ using an injection volume of 20 µl. The pretreatment of the urine or serum samples consisted of diluting steps or deproteinizing steps using perchloric acid, respectively.

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Introduction

Khellin (KHL), 5,8-dimethoxy-2-methyl-4,5-furo-6,7-chromone and visnagin (VSNG), 5-methoxy-2-methyl-4,5-furo-6,7-chromone are obtained as the furochromone constituent from *Ammi visnaga* Lam. (Umbelliferae) fruits. Recently, KHL was used as a phototherapeutic agent against psoriasis and vitiligo.¹ A number of procedures have already been described for the measurement of KHL in fruits, pharmaceutical formations, or biological samples, such as spectrophotometry,² fluorometry,³ gas chromatography^{4,5} and HPLC.⁶⁻⁹ For human urine and serum, these methods require a pretreatment as a solvent extraction and/or lack of sensitivity. In the present study, it was found that the KHL fluorescence occurred by photoirradiation with hydrogen peroxide and ethanol. In order to achieve an HPLC postcolumn photoirradiation system, the conditions of the separation and photoreaction were developed. The assay allowed easy measurements of KHL in urine and serum only by dilution and deproteinization as a pretreatment.

Experimental

Materials

Khellin was obtained from Sigma (St. Louis, USA). Other chemicals were purchased from Wako (Tokyo, Japan). A freeze drying pooled serum (Consera®) was obtained from Nissui Seiyaku (Tokyo, Japan).

Chromatographic system

The chromatographic system consisted of a high-pressure pump (Model LC 10-AS, Shimadzu, Kyoto, Japan), a pump for the postcolumn reagent (Model 5SK25GK-A, Oriental, Tokyo, Japan), a valve injector (Model E1E005, Shimamura, Tokyo, Japan) fitted with a 100 µl loop, an analytical column, a photoreactor, a fluorescence monitor (Model F-1000, Hitachi,

Tokyo, Japan) with a 12-µl flow cell, and a Multi-Pen Recorder (Rikadenki, Tokyo, Japan). In this system, the analytical column (250 × 4.6 mm i.d.) was packed with Capcell Pak C₈ (particle size 5 µm, type SG-120, Shiseido, Tokyo, Japan). Chromatography was performed at ambient temperature. The photoirradiation was carried out in PTFE tubing (0.25 mm i.d. × 1.59 mm o.d., 18 m long), which was wound around an FL-20SBL black light (20-W electric power; NEC, Tokyo, Japan). The fluorescence of the effluent solution was measured with excitation and emission wavelengths of 378 and 480 nm, respectively.

Conditions of the mobile phase and postcolumn reagent

The mobile phase, which consisted of a 40% (v/v) ethanol solution containing 75 mmol l⁻¹ hydrogen peroxide, was delivered at a flow rate of 0.5 ml min⁻¹. The postcolumn reagent was a mixture of 70 mmol l⁻¹ KH₂PO₄-70 mmol l⁻¹ NaOH (pH 12)/ethanol (1/1, v/v) at a flow rate of 0.4 ml min⁻¹.

Analytical procedure

Human urine was diluted 10 times with distilled water. The sample was filtrated using a Chromatodisc 25 A (Kurabou, Osaka, Japan). An aliquot (20 – 100 µl) of filtrated samples was injected into the present system. For a serum sample, 500 µl methanol was added to 100 µl serum in a polypropylene tube (1.5 ml) and the mixture was vortex mixed. The mixture was centrifuged at 9600 × g for 1 min and a 60 – 120 µl aliquot of the supernatant was injected into the chromatograph.

Results and Discussion

Fluorescence reaction upon irradiation

Figure 1 shows the excitation (Ex.) and emission (Em.) spectra of the fluorescence produced from khellin upon UV irradiation in the presence of hydrogen peroxide. The Ex. and Em. maxima were at 378 nm and 480 nm, respectively

Fig. 1 Fluorescence excitation (a) and emission (b) spectra of khellin obtained by a manual method. a_1 and b_1 : khellin solution; a_2 and b_2 : sample blanks for a_1 and b_1 . Reaction conditions: to 0.5 ml of khellin solution ($3.5 \mu\text{g ml}^{-1}$) was added 2.5 ml of the postcolumn reagent. The mixture was irradiated with black light FL-15BL for 20 min.

Fig. 2 Effect of the coil length on the fluorescence intensity. The photoirradiation was carried out as described in the Experimental section. The amount of khellin was 9.6 ng.

Fig. 3 Chromatograms obtained with (a) khellin and (b) visnagin. The injection volume was 20 μl . The applied concentration of khellin and visnagin was 480 ng ml^{-1} .

(visnagin: Ex. 325 nm, Em. 450 nm). Although the natural fluorescence maxima of khellin was altered by various organic solvents,¹⁰ they were in disagreement with the maxima of the

Table 1 Effect of organic modifiers on the fluorescence intensity of khellin

Organic modifier (50 v/v%)	Relative fluorescence intensity, % (Ex. 378 nm, Em. 480 nm)
None	5
Methanol	83
Ethanol	100
1-Propanol	54
2-Propanol	60
2-Methoxyethanol	43
Acetonitrile	7

present method. The fluorophore of khellin in the presence of hydrogen peroxide remains unknown.

Optimization of the chromatographic system

Table 1 lists the organic solvents examined as postcolumn reagents. Ethanol gave the highest fluorescence intensity at concentrations in the range of 45–55% (v/v). Therefore, 50% (v/v) of ethanol was added to the postcolumn reagent. The pH for the maximum fluorescence intensity in the postcolumn reagent was observed at 12.7. To prevent foam in the alkali reagent, hydrogen peroxide was added to the mobile phase. A Capcell Pak C8 was then used to obtain a peak with a good shape with the mobile phase containing 40% (v/v) ethanol. Figure 2 shows the length of PTFE tube around the 20-W black light. An unirradiated sample showed no fluorescence. The optimum conditions for photo-derivatization were found to be 75 mmol l^{-1} for the concentration of hydrogen peroxide on PTFE tubing of 15–20 m long; 18 m was adopted for further experiments.

Calibration and precision

The calibration graph was linear over the range of 1.3–52.4 ng. The correlation coefficient was 0.999, the slope was 3.8 and the intercept was 2.0 using a least-squares regression analysis. The relative standard deviation of the standard khellin was 2.1% at 328 ng ml^{-1} ($n = 10$) by injecting a volume of 20 μl . The detection limits of khellin in urine and serum ($S/N = 3$) were 32.5 and 65.0 ng ml^{-1} , respectively. The serum khellin levels following the administration of 60 mg ranged from 0.1 to 1.7 $\mu\text{g ml}^{-1}$, reported by El-Yazigi and Said,⁶ and its detection ranges in urine and serum were 1–20 and 0.1–5 $\mu\text{g ml}^{-1}$. Therefore, the sensitivity of presented method suffices for the determination of khellin in serum and urine.

Selectivity and recovery

The fluorescence intensities (Ex. 378 nm, Em. 480 nm) of the related compounds (visnagin, kynurenic acid,¹¹ kynurenine,¹² chromone, disodium cromoglycate¹³) were lower than that of khellin, and were eluted faster than the khellin peak, except for visnagin. Khellin and visnagin are analogous compounds in the fruit or preparation, their separation is necessary. Figure 3 shows standard chromatograms of khellin and visnagin. The retention times of khellin and visnagin were 18.4 and 21.6 min, respectively. Figures 4 and 5 show chromatograms of authentic khellin and urine or serum samples, respectively. The khellin peak is shown in chromatograms (a) and (b) at the corresponding retention times, whereas chromatogram (c) of the blank samples shows no peak at that position. A comparison of the chromatograms demonstrates the specificity of the present method. The recoveries ($\pm$ S.D.) of khellin added to a 24-h urine sample ($3.10 \mu\text{g ml}^{-1}$) was $95.5 \pm 2.4\%$ ($n = 6$). The

Fig. 4 Chromatograms obtained with a standard and the urine samples. (a) Standard of khellin (6.4 ng); (b) urine sample spiked with khellin; (c) blank urine. Analytical procedure and conditions as described in the Experimental section. The injection volume was 20 μ l.

recoveries ($\pm$ S.D.) of khellin added to serum sample ($0.52 \mu\text{g ml}^{-1}$) was $96.6 \pm 2.7\%$ ($n = 6$).

The present system is both sensitive and a simplified procedure for a pretreatment to estimate khellin in human urine and serum. This method should be useful in biochemical and pharmacological studies.

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Fig. 5 Chromatograms of khellin and serum. (a) Standard khellin (5.2 ng); (b) serum sample spiked with khellin; (c) blank serum. Analytical procedure and conditions as described in the Experimental section. The injection volume was 60 μ l.