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FLUORIMETRIC DETERMINATION OF KYNURENINE IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH POST-COLUMN PHOTOCHEMICAL REACTION WITH HYDROGEN PEROXIDE

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SUMMARY

A high-performance liquid chromatographic method involving post-column photochemical reaction and fluorimetric detection has been developed for the determination of kynurenine in serum. Kynurenine was separated on a column of Capcell Pak C_{18} (resistant to pH 10). The mobile phase consisted of 0.05 *M* $Na_2B_4O_7$–0.1 *M* KH_2PO_4 buffer (pH 8.5)–ethanol (97:3, v/v) containing 60 m*M* hydrogen peroxide. The post-column reagent, containing 60% (v/v) ethanol, was mixed with the mobile phase, which was irradiated with ultraviolet light to induce fluorescence. The recovery of kynurenine was 95.9 ± 5.0% ($n=6$). The method allows the determination of as little as 2 pmol of kynurenine.

INTRODUCTION

Kynurenine (KY) is a metabolite of tryptophan and its concentration in biological fluids has been reported to fluctuate in relation to various diseases such as a haemoblastic disease [1], bladder cancer [2] and vitamin B_6 deficiency [3]. Recent studies have shown that the treatment of human fibroblasts with interferon-γ induced indoleamine 2,3-dioxygenase activity followed by an increase in N-formylkynurenine and KY levels [4]. Consequently, the serum KY level in biological samples is expected to be indicative of the biochemical mechanism underlying these diseases. There have been some reports on the spectrophotometric [5, 6], fluorimetric [7], gas chromatographic [8] and mass fragmentographic [9] determination of KY in biological fluids and tissues, but they were time consuming and had only poor selectivity, except for the mass fragmentographic method. However, the last technique requires sophisticated, expensive equipment. High-performance liquid chromatography (HPLC) gives a high separation efficiency

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in the minimum time with excellent specificity for the analysis of various biological materials. HPLC has been reported with spectrophotometric [10–15], fluorimetric [16] and electrochemical detection [17].

In this paper we report the reaction of hydrogen peroxide with KY to give fluorescence on irradiation with UV light. The photochemical reaction was coupled with HPLC to achieve the micro-determination of serum KY.

EXPERIMENTAL

Chemicals

Kynurenine and kynurenic acid were purchased from Sigma (St. Louis, MO, U.S.A.). Pooled serum (Consera) was obtained from Nissui Seiyaku (Tokyo, Japan). All other chemicals were purchased from Wako (Osaka, Japan).

Equipment

Fig. 1 shows the reversed-phase HPLC system for post-column photochemical reaction. It consists of a Sanuki SSP pump (Model DM-3U 2047; Sanuki Kogyo, Tokyo, Japan), a sample injector (Model VMD-EIE 005; Shimamura Keiki Seisakusho, Tokyo, Japan) fitted with a 20-μl loop and a stainless-steel column (250×4.6 mm I.D.) packed with Capcell Pak C_{18} (particle size 5 μm, stable over the pH range 2–10; Shiseido, Tokyo, Japan) or Unisil Q C_{18} (particle size 5 μm; Gasukuro Kogyo, Tokyo, Japan), operated at room temperature. The post-column reagent containing ethanol is delivered with a Model 5SK25GK-A pump (Oriental, Tokyo, Japan). A Model RF-530 fluorescence HPLC monitor (Shimadzu, Kyoto, Japan) with a 12-μl flow cell and a Shimadzu Chromatopac C-R3A recorder–integrator are used.

The photochemical reaction was carried out in a PTFE tube (10 m×0.5 mm I.D.×1.5 mm O.D.; Gasukuro Kogyo), which was wound around a black light (Model FL-15BL; Nippon Denki, Tokyo, Japan). The black light emits characteristic wavelengths between 300 and 400 nm and has an electric power of 15 W.

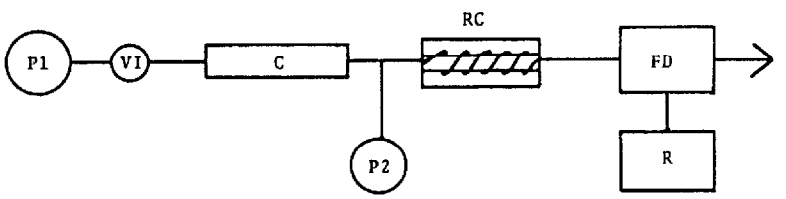

Fig. 1. Flow diagram of the HPLC system for the photochemical detection of kynurenine. P1 = Pump for mobile phase, 0.05 *M* sodium tetraborate +0.1 *M* potassium dihydrogenphosphate buffer (pH 8.5)-ethanol (97:3, v/v) containing 60 m*M* hydrogen peroxide; P2 = pump for post-column reagent, 0.1 *M* sodium carbonate +0.1 *M* boric acid containing 0.1 *M* potassium chloride buffer–ethanol (40:60, v/v) (pH 11); flow-rates, 0.8 ml/min; VI = valve injector; C = separation column (250×4.6 mm I.D.) packed with Capcell Pak C_{18} (particle size 5 μm); RC = reaction coil (PTFE tube, 10 m×0.5 mm I.D.×1.5 mm O.D.); light source, FL-15BL black light; FD = fluorescence detector (excitation 370 nm, emission 465 nm); R = recorder and integrator.

Chromatography

The mobile phase, which consisted of 613 ml of 0.05 *M* sodium tetraborate and 387 ml of 0.1 *M* potassium phosphate buffer (pH 8.5)–ethanol (97:3, v/v), containing 60 m*M* hydrogen peroxide, was delivered at a flow-rate of 0.8 ml/min. The post-column reagent (pH 11) was a mixture of 757 ml of 0.1 *M* sodium carbonate and 243 ml of 0.1 *M* boric acid containing 0.1 *M* potassium chloride–ethanol (4:6, v/v) at a flow-rate of 0.8 ml/min. The fluorescence was measured with excitation and emission wavelengths of 370 and 465 nm, respectively. The retention time of KY was about 11 min.

Sample preparation

To 100 μl of serum were added 50 μl of 1.5 *M* perchloric acid and the mixture was vortex mixed. The mixture was centrifuged at 9600 *g* for 1 min and a 20-μl aliquot of the supernatant was injected into the chromatograph.

RESULTS AND DISCUSSION

Fig. 2 shows chromatograms of KY obtained with Capcell Pak C_{18} with Unisil Q C_{18} columns. In a previous study [16], sodium sulphate was added to the mobile phase in order to suppress the tailing of the KY peak. However, this procedure cannot be applied here as it is liable to cause salt precipitation because of the high ethanol concentration in the post-column reagent. The tailing of KY can be suppressed by using a mobile phase of pH 8.5. Therefore, the packing material utilized was Capcell Pak C_{18}, which can be used up to pH 10.

Fig. 3 shows the excitation and emission spectra of KY and kynurenic acid in the presence of hydrogen peroxide exposed to UV light. The excitation maxima were at 262, 370 and 385 nm and the emission maximum was at 465 nm. In a recent paper [18] the micro-determination of serum kynurenic acid following a

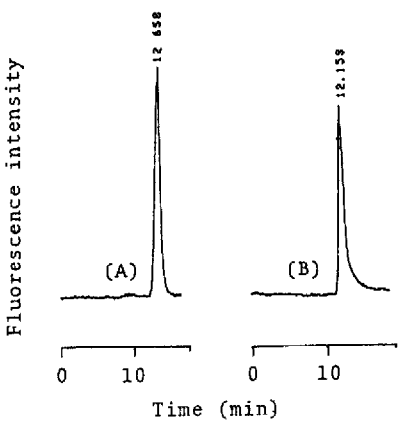

Fig. 2. Effect of pH of the mobile phase and column packing on peak tailing of kynurenine. (A) Capcell Pak C_{18} (250×4.6 mm I.D.) (5 μm), mobile phase as in Fig. 1; (B) Unisil Q C_{18} (250×4.6 mm I.D.) (5 μm), mobile phase 0.05 *M* KH_2PO_4–ethanol (95.5, v/v). Amount of kynurenine, 70 pmol.

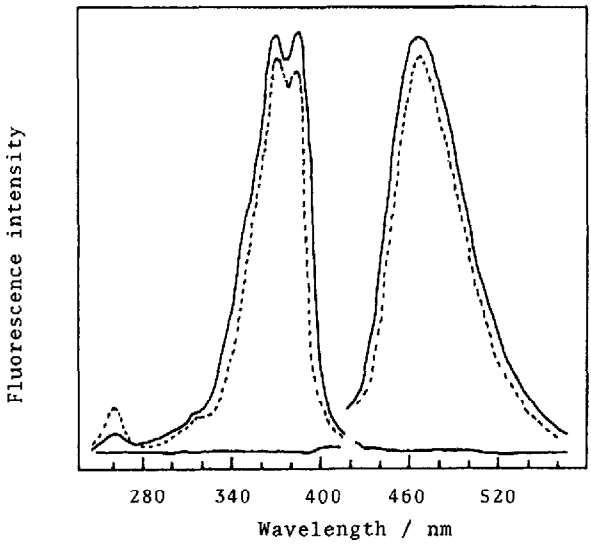

Fig. 3. Fluorescence excitation and emission spectra of kynurenine and kynurenic acid obtained by means of photochemical reaction with hydrogen peroxide. Solid lines, kynurenine (5 0 nmol/ml); broken lines, kynurenic acid (1.1 nmol/ml).

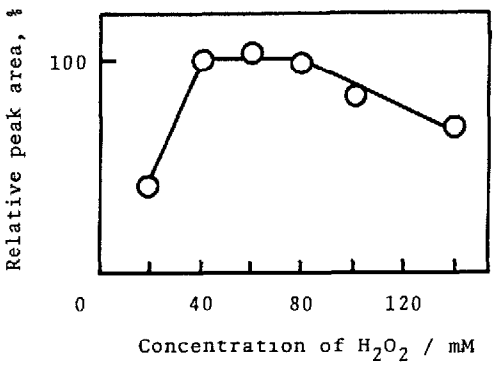

Fig. 4. Effect of concetration of hydrogen peroxide on peak area. Photochemical reaction was carried out by flow injection analysis with the apparatus described in Fig. 1, except for the concentration of hydrogen peroxide and omission of the column.

photochemical reaction with hydrogen peroxide was reported. The spectrum of KY is similar to that of kynurenic acid. Fig. 4 shows the effect of hydrogen peroxide concentration on the fluorescence intensity, the maximum fluorescence being observed at a concentration of 60 m*M*. The addition of hydrogen peroxide to the mobile phase did not affect the separation of KY.

Methanol, ethanol, 1-propanol, 2-propanol and acetonitrile were examined as post-column reagents. These organic solvents, except acetonitrile, increased the fluorescence intensity at concentrations in the range 10–50% (v/v). Ethanol gave the highest fluorescence intensity at concentrations in the range 50–65% (v/v). Therefore, 60% (v/v) of ethanol was added to the post-column reagent.

Fig. 5 shows the effect of the pH of the post-column reagent on fluorescence intensity. The optimum pH range was 10.5–11.5 and therefore pH 11.0 was adopted. Fig. 6 shows the effect of the length of the PTFE tube around the 15-W black light. The best results were obtained with a tube of length 10 m. The calibration graph was linear over the range 2–400 pmol; the correlation coefficient was 0.9998, the slope was 6.45 and the intercept −0.924. The within-day relative standard deviation at 20 pmol of KY was 1.9% ($n=12$).

Fig. 7 compares the chromatograms of UV-irradiated (A) standard, (B) KY added to Consera and (C) Consera, and the bottom trace was obtained without

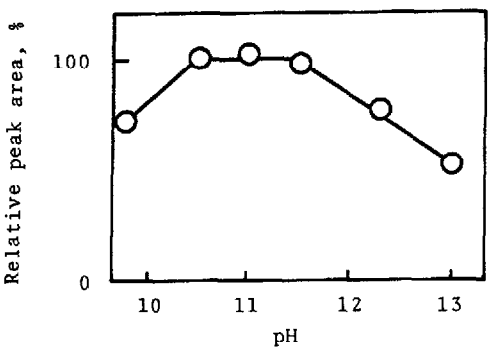

Fig 5. Effect of pH of the post-column reagent on peak area. Photochemical reaction was carried out as described in Fig. 1, except for the pH. Amount of kynurenine, 50 pmol.

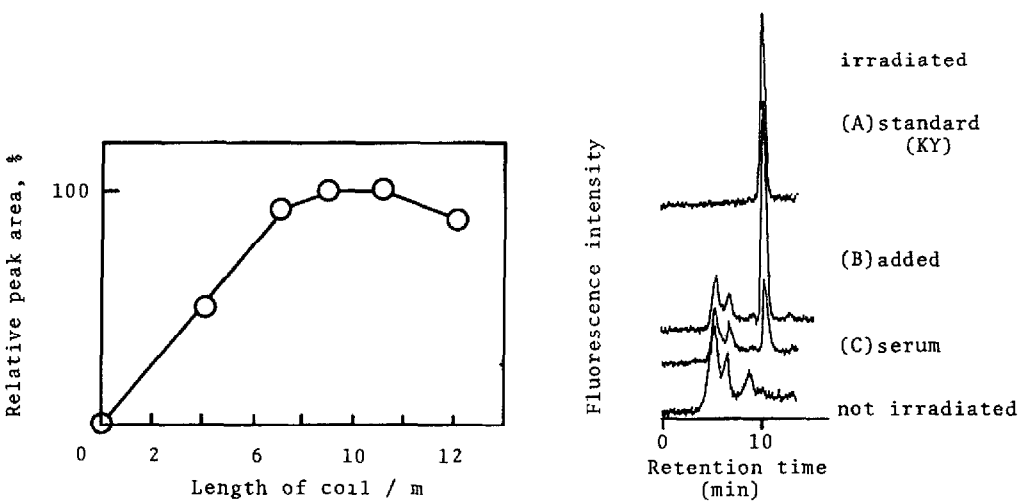

Fig. 6. Effect of length of PTFE tube on peak area. Photochemical reaction was carried out as described in Fig. 1, except that the separation column was omitted. Amount of kynurenine, 50 pmol.

Fig. 7. Chromatograms of kynurenine and Consera. Irradiation with UV light: (A) standard kynurenine; (B) kynurenine added to Consera; (C) Consera Bottom trace, no irradiation with UV light. HPLC conditions as described in Fig. 1. Amounts of standard kynurenine and serum kynurenine, 84 and 30 pmol, respectively.

TABLE I

PRECISION OF THE ASSAY OF KYNURENINE IN SERUM

Precision	Concentration (mean ± S.D., $n=6$) (nmol/ml)	Relative standard deviation (%)
Within-day	1.46 ± 0.076	5.2
Between-day	1.52 ± 0.098	6.4

irradiation. Chromatograms B and C show a sharp peak with a retention time identical with that of the standard KY, whereas the chromatogram of the unirradiated sample shows no peak at this position. Comparison of the chromatograms demonstrates the specificity of the proposed method.

The peak of KY was confirmed by using a post-column reaction as described previously [16]. The equipment and the mobile phase used were the same as in Fig. 1. The post-column reagent was a mixture of sodium carbonate and sodium hydroxide and the post-column reaction was carried out at 60°C with a 10-m reaction tube. The fluorescence was measured at excitation and emission wavelengths of 315 and 385 nm, respectively, and the following results were obtained. The concentration of KY in standard serum was 1.38 ± 0.192 nmol/ml ($n=6$), which is in agreement with those obtained by post-column photochemical reaction, i.e., 1.46 ± 0.076 nmol/ml ($n=6$). In the absence of hydrogen peroxide KY did not give fluorescence. In 1984, we have detected KY after the removal of hydrogen peroxide from the mobile phase. In the present work, we developed the procedure in which the removal of hydrogen peroxide is not necessary.

The recovery (± standard deviation) of KY added to standard serum (4.2 nmol/ml) was 95.9 ± 5.0% ($n=6$). The results of within-day and between-day assays are summarized in Table I. The concentration of KY is similar to that reported previously, 1.23 ± 0.308 nmol/ml [17] and 0.47 ± 0.1 μg/ml [15].

The photochemical reactor is very simple and easily assembled. The method is sensitive and specific enough to determine KY in human serum and is expected to be useful not only in pathological studies but also in the diagnosis of several metabolic diseases.

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