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FLUOROMETRIC DETERMINATION OF KYNURENINE DERIVATIVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH HYDROGEN PEROXIDE AND SODIUM CARBONATE

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Fluorometric method for the determination of kynurenine derivatives based on their reaction with alkaline hydrogen peroxide in the presence of copper(II) sulfate was established. Kynurenine and N-formylkynurenine can be determined at the concentration as little as 25 ng/ml. The reaction was applied to the postcolumn derivatization of kynurenines by high performance liquid chromatography(HPLC). The calibration curves for kynurenine, N-formylkynurenine and 3-hydroxykynurenine show linear relationships in the range from 1 ng to 100 ng, from 2 ng to 100 ng, and from 50 ng to 1 μg, respectively. The relative standard deviations for 10 ng of kynurenine and N-formylkynurenine were less than 3%.

Kynurenine is one of the major metabolites of tryptophan on the pathway initiated by the induced liver enzyme tryptophan pyrrolase. These metabolites have been isolated by means of ion exchange chromatography, and paper chromatography[1)-4)]. Although there are some reports on the colorimetric[5)11)12)], fluorometric[5)-10)], gas chromatographic[13)] and mass-fragmentographic[14)] determination of kynurenines in the biological fluids and tissues, only the mass-fragmentographic method has met the requirement of specificity and sensitivity. However, the availability of this sophisticated equipment is not yet satisfactory mainly because of its enormous cost. Reversed phase high performance liquid chromatographyic (HPLC) method gave high separation efficiency with minimum time of analysis and excellent specificity for the analysis of various biological materials[15)16)].

HPLC method with the detection utilizing native fluorescence or fluorescence reaction seems to be a promising tool for this purpose. We found that hydrogen peroxide reacts with kynurenines in the presence of copper(II) in alkaline media at moderate temperature to give derivatives which show intense fluorescence. This reaction was applied to the HPLC determination of 3-hydroxykynurenine, kynurenine, and N-formlkynurenine with acceptable sensitivity and precision.

EXPERIMENTAL

Kynurenine and indole derivatives were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Hydrogen peroxide was obtained from Mitsubishi Gas Chemical (Tokyo, Japan), other chemicals used were obtained from Wako Pure Chemical (Osaka, Japan). LiChrosorb RP-18 (particle size 10 μm) was purchased from E. Merck (Darmstadt, G.F.R.).

Fluorescence reagent for manual assay procedure was prepared immediately before use by mixing 10 ml of 240 mM sodium carbonate with 1 ml of 0.6 mM copper(II) sulfate and 9 ml of 50 mM hydrogen peroxide.

Preparation of urine sample

Human urine was collected for 24 h and pretreated according to Brown-Price method[1]. An aliquot of 10 ml of human urine was diluted to 120 ml with distilled water. To the diluted sample was added 30 ml of 0.1 M hydrochloric acid, and the mixture was applied to a column of Dowex 50 x 12(H^+ form, 3 cm x 1 cm I.D.). The column was washed successively with 50 ml of 0.2 M hydrochloric acid, 100 ml of 0.5 M hydrochloric acid, 420 ml of distilled water, and 50 ml of 2.4 M hydrochloric acid. Kynurenine was eluted with 100 ml of 5 M hydrochloric acid. Five milliliters of this effluent was taken and adjusted to about pH 3-4 by adding 2.1 g of sodium bicarbonate and 1 M acetic acid. This solution was filtered through 0.45 μm membrane filter.

Manual assay procedure

To 2 ml of sample solution was added 1ml of the fluorescence reagent. The mixture was incubated in a water bath for 20 min at 50°C. After cooling in running water, the fluorescence intensity of the reaction mixture was measured at 385 nm with excitation at 315 nm with Hitachi Fluorescence spectrophotometer Model 650-10S.

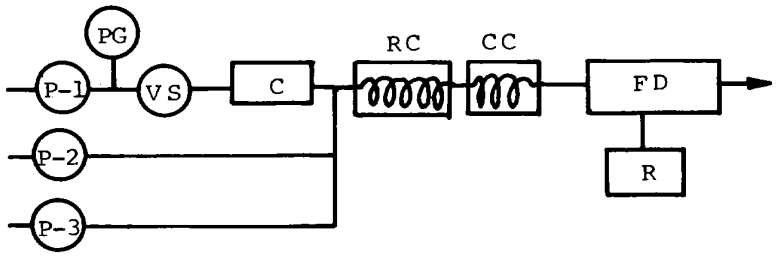

Fig. 1 Flow diagram of HPLC system for kynurenines with postcolumn derivatization using hydrogen peroxide-sodium carbonate-copper(II) sulfate reagent
P-1: high pressure pump for mobile phase; PG: pressure gauge; VS: valve universal injector; C: separation column packed with LiChrosorb RP-18 (250 mm × 4 mm I.D., particle size 10 μm); RC: reaction coil (15 m × 0.5 mm I.D., teflon) kept at 60°C in water bath; CC: cooling coil (2 m × 0.5 mm I.D.); FD: fluorescence detector; R: recorder; P-2 and P-3: reagent pumps for the delivery of hydrogen peroxide solution and sodium carbonate solution, respectively.

HPLC assay procedure using hydrogen peroxide-copper(II) sulfate reagent

Figure 1 shows schematic diagram of the chromatographic system. Separation column (250 mm x 4 mm I.D.) was packed with LiChrosorb RP-18 (particle size 10μm) by SNK High Performance Column Packer Model SPC-102 (Sanuki Kogyo, Tokyo, Japan) by slurry packing method[17] in our laboratory. The mobile phase was 50 mM acetate buffer (pH 4.1) containing 0.1 M sodium sulfate, and 10 μM copper(II) sulfate. A high pressure pump with a valve universal injector (Sanuki Kogyo, Toyo, Japan) was used to deliver mobile phase at a flow rate of 1.5 ml/min.

Postcolumn reagent solution was prepared as follows. Six milliliters of 30% hydrogen peroxide solution was diluted to 1000 ml with distilled water, and 750 ml of 0.4 M sodium carbonate-12 mM sodium hydroxide (pH 12.8) was also diluted to 1000 ml with distilled water. Flow rates of these reagents were adjusted to 0.5 ml/min. An aliquot (200 μl) of the urinary sample pretreated as described above was injected onto the separation column. The fluorescence was monitored at 385 nm with excitation at 315 nm with Hitachi Fluorescence Spectrophotometer Model 650-10S equipped with a 40 μl flow cell(SNK Model FL 101-40, Sanuki Kogyo, Tokyo, Japan).

RESULTS AND DISCUSSION

Figure 2 shows excitation and emission spectra of kynurenine reacted with hydrogen peroxide-sodium carbonate-copper(II) sulfate. Excitation and emission maxima were 315 nm and 385 nm, respectively. N-Formylkynurenine gave similar spectra, but excitation and emission maxima of 3-hydroxykynurenine were 300 nm and 385 nm, respectively.

Table 1 exhibits the effect of metal ions on the fluorescence intensity of kynurenine. The fluorescence intensity of kynurenine was found to be dependent on the kind of alkaline solution used (relative fluorescence intensity: 100 for 75 mM sodium carbonate, 23.5 for 75 mM sodium hydroxide, 11.4 for 75 mM sodium bicarbonate). The combination of copper(II) ion and sodium carbonate gave the

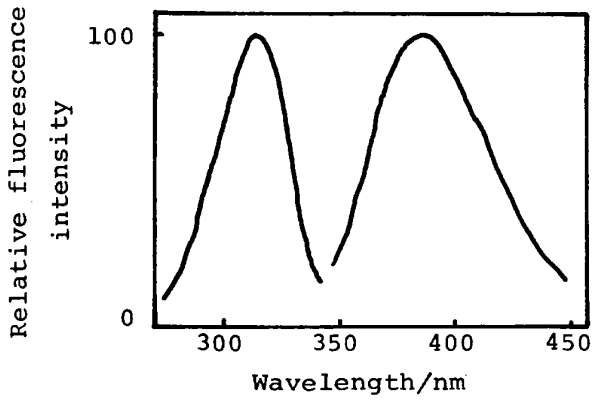

Fig. 2 Fluorescence excitation and emission spectra of kynurenine allowed to react with hydrogen peroxide-sodium carbonate-copper(II) sulfate reagent

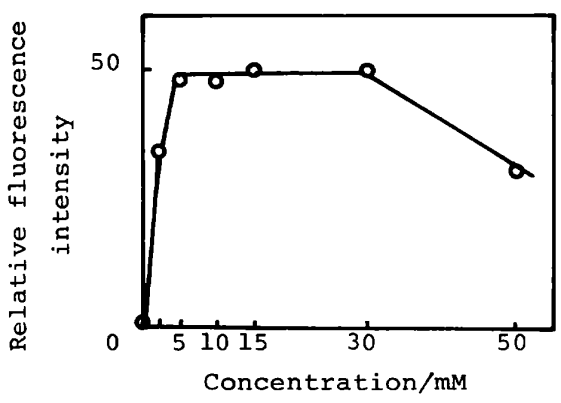

Fig. 3 Effect of concentration of hydrogen peroxide on the fluorescence intensity of kynurenine

Table 1 Effect of metal ions on the fluorescence intensity of kynurenine

Metal	Relative fluorescence intensity
Iron(III) chloride	38
Iron(II) chloride	34
Cobalt(II) chloride	25
Nikel(II) chloride	23
Copper(II) sulfate	100

most intense fluorescence. Figure 3 exhibits the effect of concentration of hydrogen peroxide on the fluorescence intensity of kynurenine. Maximum intensity was observed in the range between (5-30) mM of hydrogen peroxide. Figure 4 shows the fluorescence intensity of kynurenine as a function of reaction time at various reaction temperature. The fluorescence intensity reached to a plateau after 20 min of the reaction at 50°C. Accordingly the reaction time of 20 min was adopted in the manual assay procedure. The fluorescence developed was stable for at least 5 h at room temperature. The calibration curves for kynurenine and N-formylkynurenine were linear in the range of final concentration of 25 ng/ml to 500 ng/ml and that for 3-hydroxykynurenine in the range from 500 ng/ml to 5 μg/ml (Fig. 5). The relative standard deviation for the determination of these compounds (500 ng/ml of kynurenine and N-formylkynurenine, 5 μg/ml of 3-hydroxykynurenine, n=5 each) was less than 2%.

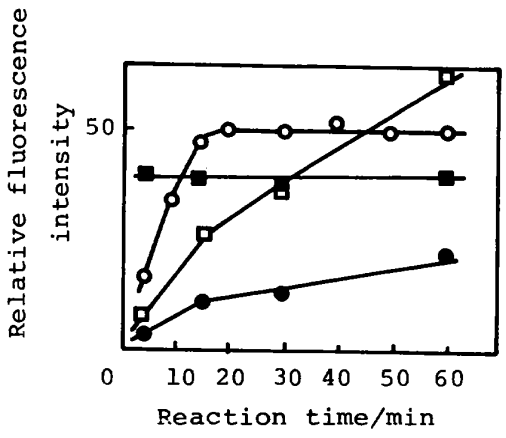

Fig. 4 Effect of reaction time and temperature on the fluorescence intensity of kynurenine
●: 22°C; □: 37°C; ○: 50°C; ■: 90°C.

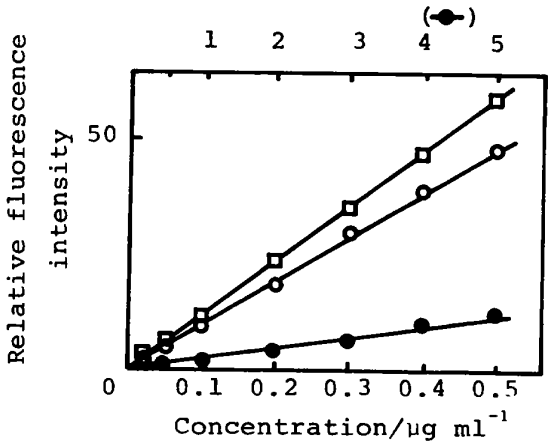

Fig. 5 Calibration curves for kynurenine obtained by manual method
□: N-formylkynurenine; ○: kynurenine; ●: 3-hydroxykynurenine.

Table 2 Effect of the addition of some compounds on the fluorescence intensity of kynurenine with hydrogen peroxide-sodium carbonate-copper(II) sulfate reagent*

Compound	Amount of compound added/μg	Relative ** intensity
None	-	100
Glucose	200	98
Sodium chloride	200	102
Sodium sulfate	200	103
Methanol	20	101
Uric acid	20	95
Acetaldehyde	20	101
Arginine	20	95
Urea	200	95
Glycine	20	63
Ascorbic acid	200	67
Tryptophan	2	118
Indole-3-acetic acid	2	119
Serotonin	2	105
5-Hydroxyindole-3-acetic acid	2	94
Kynurenic acid	2	134
Xanthurenic acid	2	109

*Amount of kynurenine: 1.5 μg

**[Intensity (+compound)/intensity (none)] × 100

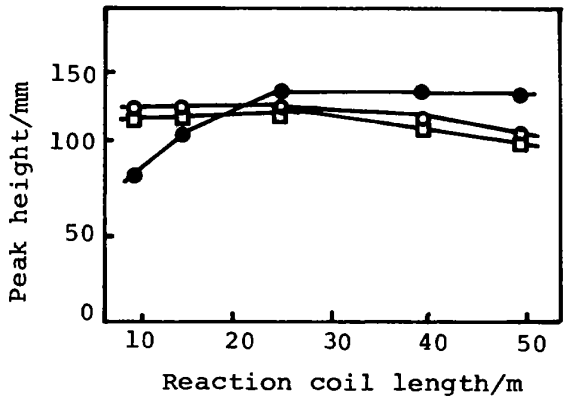

Fig. 6 Effects of reaction coil length and reaction temperature on the peak height of kynurenine

●: 50°C; ○: 60°C; □: 70°C.

Relative fluorescence intensity of kynurenine in the presence of some compounds was measured and listed in Table 2. Methanol, uric acid, acetaldehyde, and arginine did not interfere with the reaction when the same amount of these compounds as that of kynurenine was added to the sample solution. However, addition of large excess amount of these compounds showed some interference. Urea affected the reaction slightly. Ascorbic acid, glycine, tryptophan, and indole-3-acetic acid were found to affect the assay. The interference by tryptophan may be due to the formation of N-formylkynurenine. 3-Substituted indoles gave fluorescence in this reaction, whereas 5-hydroxyindoleacetic acid did not. Although kynurenic acid did not give fluorescence in this reaction, its native fluorescence affected the measurement.

The above mentioned reaction was applied to the postcolumn derivatization of kynurenines in HPLC. Fluorescence was developed when the eluate from the separation column was mixed with sodium carbonate-sodium hydroxide reagent and hydrogen peroxide reagent at 60°C. Figure 6 shows the peak height of kynurenine as a

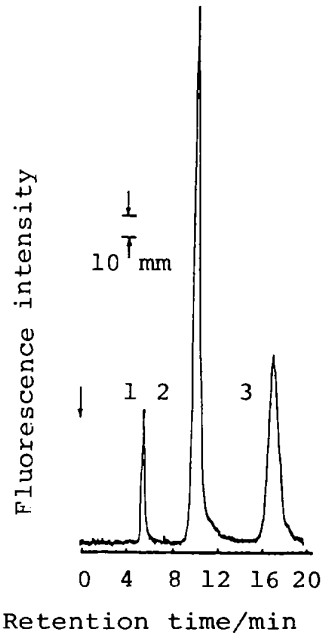

Fig. 7 Chromatogram for a mixture of authentic kynurenine with postcolumn derivatization using hydrogen peroxide-sodium carbonate-copper(II) sulfate
Column: LiChrosorb RP-8 (250 mm × 4 mm I.D., particle size 10 μm); mobile phase : 50 mM acetate buffer (pH 4.1) containing 0.1 M sodium sulfate and 10 μM copper (II) sulfate. Peaks: 1; 1μg of 3-hydroxykynurenine, 2: 83 ng of kynurenine, 3: 70 ng of N-formylkynurenine.

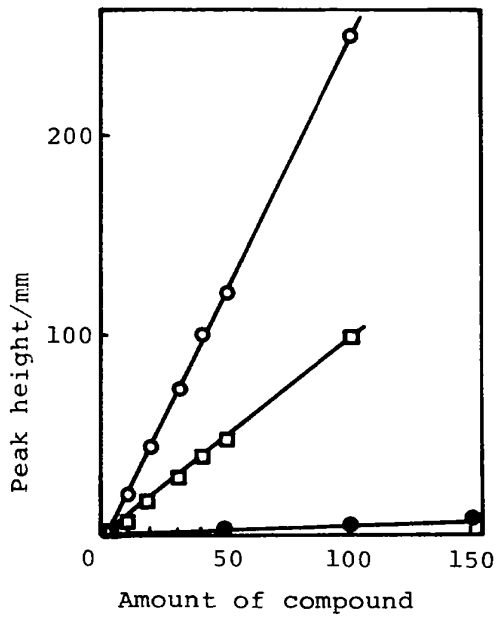

Fig. 8 Standard curves for kynurenines by HPLC method
●: 3-hydroxykynurenine; ○: kynurenine; □: N-formylkynurenine.

function of reaction temperature and reaction coil length. The peak height was at maximum at 60°C when the coil length was (10-15) m. Excellent separation was observed for tryptophan metabolites. 3-Hydroxykynurenine, kynurenine, N-formylkynurenine were eluted in this order. Figure 7 shows a chromatogram for the mixture of standards of kynurenines.

Limit of detection of 3-hydroxykynurenine, kynurenine, and N-formylkynurenine was 15 ng, 0.2 ng, and 0.4 ng, respectively. Calibration curves for 3-hydroxykynurenine, kynurenine, N-formylkynurenine were linear in the range of 50 ng to 1000 ng, 1 ng to 100 ng, 2 ng to 100 ng, respectively (Fig. 8). The relative standard deviation for 10 ng of kynurenine is 2.1% (n=5). The sensitivity is

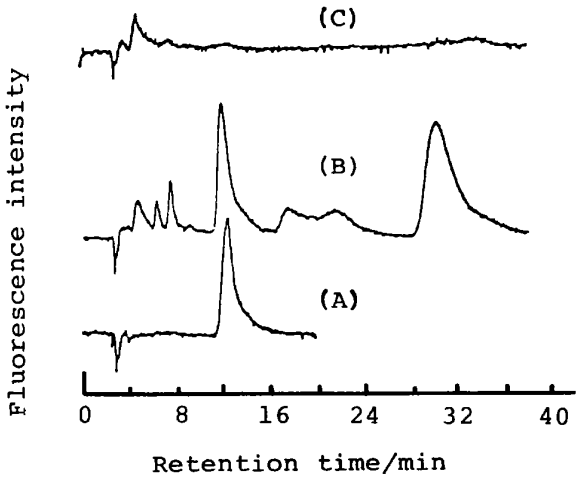

Fig. 9 Chromatograms for kynurenine
Chromatographic conditions are the same as those in Fig. 7. All samples in this figure were pretreated with ion exchange resin. (A): 20 μg of kynurenine; (B): 10 ml of urine sample; (C): 10 ml of the same urine sample as (B) reacted without hydrogen peroxide.

sufficient for the determination of kynurenine in human urine.

Figure 9 illustrates the elution profiles of kynurenine standard, a urine sample, and reagent blank (the same urine sample reacted without hydrogen peroxide reagent). Kynurenine standard was detected as a single peak, with a retention time of about 11 min (Fig. 9A). Kynurenine contents in the urine of healthy human adults collected for 24 h were as follows: 2.7 mg/day (female, 38 years), 3.5 mg/day (male, 38 years), and 1.2 mg/day (male, 30 years). Kynurenine could not be detected in these urine samples with ultraviolet absorption.

The proposed method is simple, rapid, and its application to the analysis of patient urine may be useful not only in the pathological studies but also in the diagnosis.

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Keyword phrases

fluorometic determination of kynurenine derivatives in urine by high performance liquid chromatography ; tryptophan metabolites; postcolumn derivatization with hydrogen peroxide-sodium carbonate-copper(II) sulfate.

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