

Simultaneous Determination of Kynurenine and Kynurenic Acid by High-Performance Liquid Chromatography Photoirradiation System Using a Mobile Phase Containing 18-crown-6

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ABSTRACT: A high-performance liquid chromatography (HPLC) system has been developed for the fluorometric determination of kynurenine (KYN) and kynurenic acid (KYNA) in human serum using a mobile phase containing 18-crown-6. A retention time of KYNA was adjusted with pH of phosphate buffer in 18-crown-6. KYN and KYNA were separated on a CAPCELLPAK C18 (250 × 4.6 mm i.d.). The mobile phase consisted of 35 mmol/L phosphate buffer (pH 8.0)/methanol (85/15, v/v) containing 35 mmol/L hydrogen peroxide and 10 mmol/L 18-crown-6. The retention times of KYN and KYNA were 18 and 24 minutes, respectively. The calibration graphs of KYN and KYNA were linear over the range 180 to 2900 and 1 to 84 nmol/L by injecting a 50- μ L volume of KYN and KYNA, respectively. Pretreatment of serum was achieved by deproteinization only. The mean recoveries of KYN and KYNA from serum were more than 97%.

KEYWORDS: kynurenine, kynurenic acid, 18-crown-6, post-column UV irradiation, hydrogen peroxide, fluorescence detection, high-performance liquid chromatography

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Introduction

Kynurenine (KYN) is one of the tryptophan metabolites produced by KYN pathway. Kynurenine is known as an endogenous ligand of aryl hydrocarbon receptor¹ and reportedly acts directly on glioma cells to promote tumor formation and suppress the immune response.² Kynurenic acid (KYNA) is produced by the action of KYN aminotransferase and has been reported to be the final product in mammalian brain.³ KYNA acts as an antagonist of glutamate N-methyl-D-aspartate receptor and α_7 nicotinic acetylcholine receptor at physiological concentrations and has an anti-neurotoxic effect to suppress the excitotoxicity of glutamatergic nerves.⁴ In addition, relationships with depression and schizophrenia have been reported.⁵

Therefore, the determination of KYN and KYNA in biological samples can provide useful information. Currently, a number of procedures have been described for the measurement of KYN and KYNA in biological samples, such as high-performance liquid chromatography (HPLC)^{6,7} and liquid chromatography tandem mass spectrometry (LC-MS).⁸ High-performance liquid chromatography, with native fluorescence or ultraviolet (UV) detection, lacks sensitivity and/or specificity. Although LC-MS is excellent in specificity and sensitivity, it requires complicated

maintenance such as cleaning of a sample inducing device. We have reported a fluorometric detection of KYNA or KYN using an HPLC post-column photoirradiation system.^{9,10} These methods are sensitive and is an easy procedure for pretreatment. However, in the simultaneous determination of KYN and KYNA using the simple procedure of pretreatment, it was difficult to separate KYN from coexisting components in serum sample while setting optimal retention times of KYNA (about 18 minutes). In this study, it is found that the retention time of KYNA was adjustable by changing the pH of phosphate buffer in mobile phase. This phenomenon was applied to the HPLC photoirradiation system for simultaneous determination of KYN and KYNA in serum sample.

Experiment

Chemicals

L-Kynurenine and kynurenic acid were purchased from Sigma-Aldrich (St. Louis, MO). Disodium hydrogen phosphate, potassium dihydrogen phosphate, hydrogen peroxide, and 18-crown-6 were purchased from FUJIFILM-Wako Pure Chemicals Co (Osaka, Japan). Freeze-dried serum (Consera[®]), pooled serum for quality control, was obtained from Nissui Seiyaku (Tokyo, Japan).



Fluorescence spectra of UV-irradiated KYN and KYNA

Fluorescence spectra were recorded by using a fluorescence spectrophotometer (F-7000; HITACHI, Tokyo, Japan). Reaction conditions: to 0.5 mL of KYNA or KYN solutions (30 µg/mL) was added to 2.5 mL of the mobile phase. The mixture was irradiated with UV light (a black light, Model FL-15BL) for 20 minutes.

Chromatographic system

The chromatographic system comprises a high-pressure pump (Model LC-20AT; SHIMADZU, Kyoto, Japan), a sample injector (Model 7725i; Rheodyne, Berkeley, USA), and an analytical column (250 × 4.6 mm i.d.) packed with CAPCELLPAK C18 MG II (particle size 5 µm, OSAKA SODA, Osaka, Japan). A Model RF-20Axs Fluorescence Detector (SHIMADZU, Kyoto, Japan) and a Chromato-PRO (Run Time Co, Tokyo, Japan) recorder-integrator were used. Ultraviolet irradiation was performed in an co-polymer of ethylene-tetrafluoroethylene (ETFE) tube (10 m × 0.25 mm i.d. × 1.5 mm o.d.), which was wound around a "black light" source (Model FL-15BL; NEC, Tokyo, Japan). The mobile phase consisted of 35 mmol/L potassium dihydrogen phosphate-disodium hydrogen phosphate buffer (pH 8.0) containing 35 mmol/L hydrogen peroxide, 10 mmol/L 18-crown-6 (CE), and 15% methanol and was delivered at a flow rate of 0.8 mL/min at room temperature. The fluorescence was measured with excitation of 370 nm and emission of 465 nm.

Analytical validation

Calibration graphs were based on the analysis of standard solution of KYN or KYNA with injection amounts of 180 to 2900 and 1 to 84 nmol/L by injecting a 50-µL volume of KYN and KYNA, respectively. The detection limit was determined as three times the baseline noise. Intra- and inter-day precisions for the developed method were measured in terms of relative standard deviation (%) with 180 nmol/L KYN or 5 nmol/L KYNA. To determine the recovery, serum sample with Consera was prepared by adding each 0.5 mL of 2560 nmol/L KYN and 104 nmol/L KYNA standard solution.

Pretreatment of serum

After addition of 3-mL distilled water in freeze-dried serum (Consera), allowed to stand for 20 minutes, then it was prepared by stirring. To 200 µL of the serum, 100 µL of 1.5 mol/L perchloric acid was added. After the mixture was mixed in a vortex mixer, it was centrifuged at 9600g for 1 minute. Following added to 100 µL of 1.5 mol/L potassium chloride, centrifuged for 1 minute, an aliquot (50 µL) of the supernatant was injected into the chromatograph.

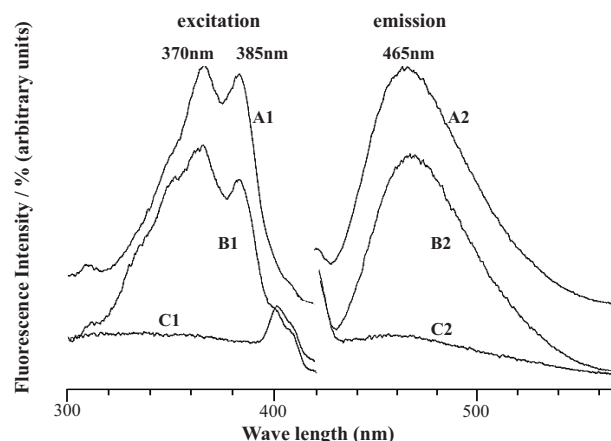


Figure 1. Fluorescence excitation and emission spectra of KYN and KYNA by a manual method: (A1) and (A2): KYNA solution; (B1) and (B2): KYN solution; and (C1) and (C2): blank for KYNA and KYN.

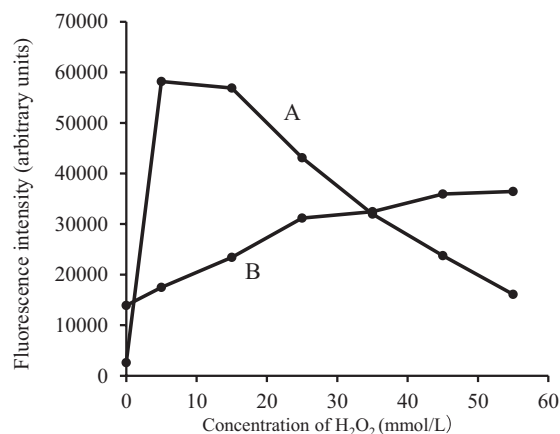


Figure 2. Effect of H₂O₂ concentration on the fluorescence intensity: (A) KYNA (amount was 4.8 nmol/L) and (B): KYN (amount was 850 nmol/L).

Results and Discussion

Fluorescence spectra of UV-photoirradiated KYN and KYNA

Figure 1 shows the excitation and fluorescence spectra of the KYN and KYNA. The spectra of KYN are similar to that of KYNA. Therefore, this method was set up at excitation 370 nm and emission 465 nm.

Mobile phase conditions

Addition of methanol to the mobile phase increased the fluorescence intensity of KYN.¹⁰ Therefore, 15% v/v was added to the mobile phase.

Figure 2 shows the effect of hydrogen peroxide concentration in the mobile phase. The fluorescence intensity of KYNA was maximum in 5 mmol/L, while that of KYN decreased. The sensitivity of KYNA was higher than that of KYN; therefore, 35 mmol/L was adopted.

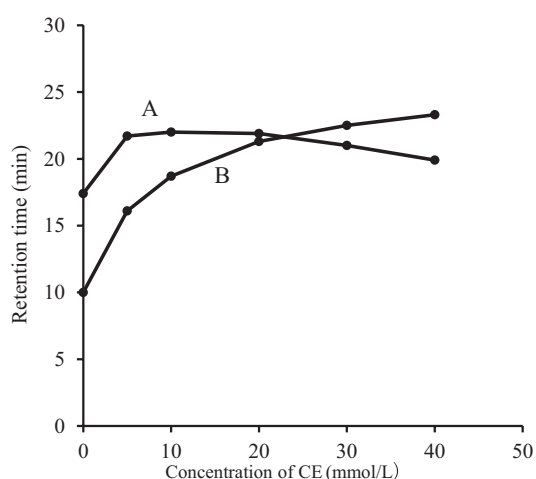


Figure 3. Effect of CE concentration on the retention time: (A) KYNA (amount was 4.8 nmol/L) and (B) KYN (amount was 850 nmol/L).

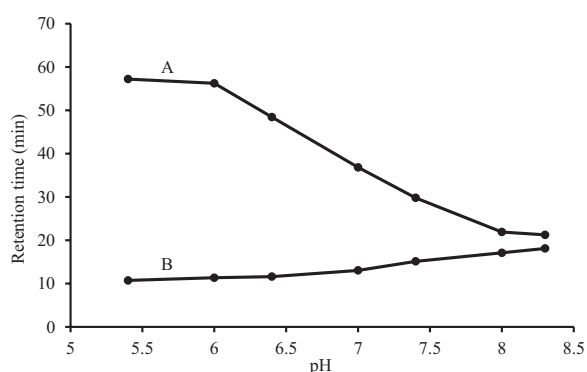


Figure 4. Effect of pH in phosphate buffer on the retention time: (A) KYNA (4.8 nmol/L) and (B) KYN (850 nmol/L).

Figure 3 shows the retention times of KYNA and KYN with 18-crown-6 (CE). The addition of 10 mmol/L CE is adequate to separate the compounds.

Figure 4 shows the retention time of KYNA and KYN. The retention time of KYN was 10 minute and that of KYNA was 58 minute at pH 5.4, and then 19 and 21 minutes at pH 8.4, respectively. The retention time of KYNA was not decreased without CE. It has been reported that the retention time usually becomes longer by the addition of CE in mobile phase.^{11,12} The mechanism of reduction on the retention time is still not clear; however, it is speculated that the ion of phosphate buffer affects the retention time of KYNA. For separate of KYN and KYNA from components in the serum, the pH of phosphate buffer was adopted to 8.0, which gave retention times for KYN and KYNA of 18 and 24 minutes, respectively.

Table 1. Validation results for KYN and KYNA.

	KYNURENINE	KYNURENIC ACID
Linear range (nmol/L)	180-2900	1.0-84
Regression equation	$y=29x-933$	$y=3029x-1075$
Correlation coefficient (r)	0.999	0.999
Detection limit (nmol/L) ^a	34	0.1
Intra-day precision, %RSD (n=6)	1.43	1.41
Inter-day precision, %RSD (n=6)	6.41	4.63
Recovery (%) (n=6) ^b	100.5 ± 0.9	99.7 ± 1.7

^aSignal to noise=3.

^bMean values ± standard deviation.

Validation of the chromatographic system

Table 1 shows the validation results for this method. The intra- and inter-day precisions for KYN and KYNA were both <7% (n=6). The recovery (%) of KYN and KYNA were both >97% (n=6).

Determination of KYN and KYNA in human serum

Figure 5A shows a chromatogram of the pooled serum sample but without CE in the mobile phase; it was difficult to separate the KYN from the impurities. Figure 5B shows the separation of KYN and KYNA from the impurities after addition of 10 mmol/L of CE to the mobile phase. Figure 5C shows a chromatogram of the pooled serum spiked with the standards. The KYN and KYNA peaks are shown in Figure 5B and C, respectively. The amounts of KYN and KYNA in pooled serum were 318 ± 8.0 (n=6) nmol/L and 12.7 ± 0.6 (n=6) nmol/L, respectively. These values in pooled serum were lower than the literature value (KYN, 1790 ± 440 nmol/L; KYNA 28.4 ± 2.16 nmol/L);^{13,14} however, the values of human serum, Figure 5D, were 1200 and 29.5 nmol/L, respectively. The difference of KYN and KYNA concentration were considered to use a freeze-dried serum.

Conclusion

For the simultaneous determination of KYN and KYNA, HPLC post-column photoirradiation using the mobile phase containing 18-crown-6 has been developed. The retention time of KYNA was adjusted by alterations in the component of phosphate buffer containing 18-crown-6. This method is sensitive and simplified the procedure of pretreatment and should be useful in biochemical studies.

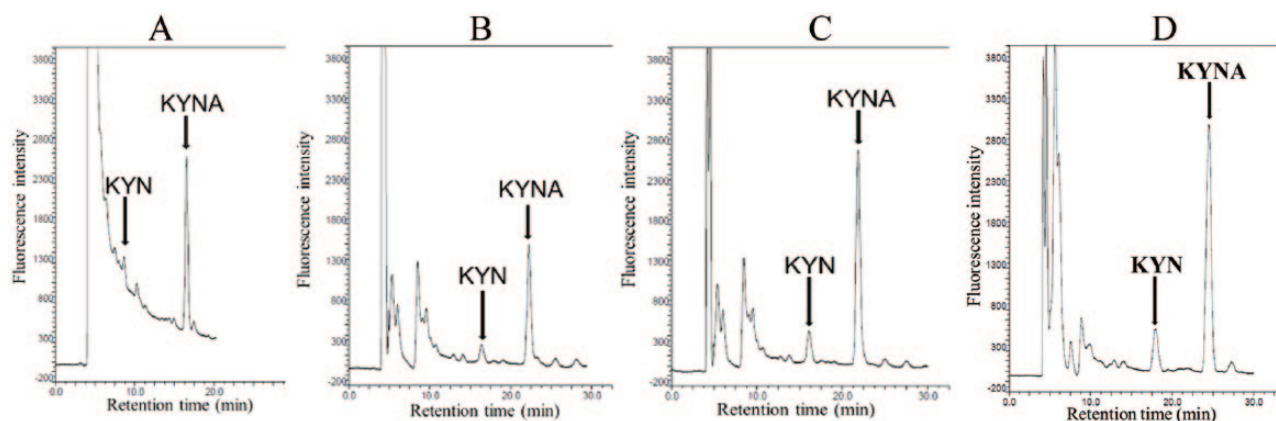


Figure 5. Chromatograms of KYN and KYNA in pooled serum and human serum: (A) without 18-crown-6 in mobile phase, (B) with 18-crown-6 in mobile phase, (C) serum sample spiked with KYN (320 nmol/L) and KYNA (13 nmol/L) under the same condition as (B), and (D) human serum.

Author Contributions

MA designed the study, and wrote the initial draft of the manuscript. KM contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All other authors have contributed to data collection and interpretation. All authors approved the final manuscript.

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