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Note

Fluorimetric micro-determination of kynurenic acid, an endogenous blocker of neurotoxicity, by high-performance liquid chromatography

KATSUMI SHIBATA

Laboratory of Nutritional Biochemistry, Department of Food Science and Nutrition, Teikoku Women's University, Moriguchi, Osaka 570 (Japan)

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Stone [1] has reported that kynurenic acid (KA), a tryptophan metabolite, is one of the most effective blockers of excitatory amino acid mediated synapses presently available. KA is able to antagonize the responses of cortical neurons to ionophoretically applied N-methyl-D-aspartate, quisqualate, quinolinate [2] and kainate [3]. The inhibitory effects of KA on neurotoxicity are considered to be primarily due to interference with the post-junctional activity of a transmitter, rather than a direct depressant action on the post-synaptic cell [4]. Altered concentrations of tryptophan metabolites in blood and/or urine have been also observed in depression [5], schizophrenia [6], carcinoid tumours [7] and alcoholism [8].

Various methods have been used to determine tryptophan and its metabolites in physiological fluids. Recently, mainly high-performance liquid chromatographic (HPLC) methods have been used [9–14]. Krstulović et al. [14] reported a reversed-phase liquid chromatographic method for concurrent measurement in serum of tryptophan and eight of its metabolites, including KA. These were separated by gradient elution and detected both by UV absorption at 254 nm and amperometrically at an oxidation potential of +700 mV versus Ag/AgCl. Many of the tryptophan metabolites are amperometrically active, but KA is not. Because the concentration of KA in biological fluids is low, any methods for its detection must be highly sensitive; KA is not fluorimetrically detectable at its endogenous concentration [14]. Iinuma et al. [15] reported that the fluorescence of KA increased in the presence of Zn^{2+}. In their method KA was separated using a Dowex 50W-X12 (H^+ form, 200–400 mesh) column as reported by Satoh and

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Price [16]. This chromatography is tedious, time-consuming and requires particular skills.

This paper describes a practical HPLC micro-determination method for KA. The fluorescence of KA separated on a reversed-phase column is increased about 50-fold with 1 *M* zinc acetate as the post-column reagent. The detection limit is 0.2 pmol (41.8 pg).

EXPERIMENTAL

Chemicals

KA was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Zinc acetate was obtained from Wako (Osaka, Japan). All other chemicals used were of the highest commercial grade available.

Liquid chromatography

HPLC was performed on a Shimadzu LC-4A system with a Shimadzu RF-540 spectrofluorophotometer, equipped with an LC flow-cell unit (Shimadzu, Kyoto, Japan), a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), and a 5-ODS-H column (150 mm×4.6 mm I.D., particle size 5 μm, Chemco Scientific, Osaka, Japan). A degassed solution of 10 m*M* sodium acetate–acetic acid buffer (pH 4.5) and acetonitrile (95:5, v/v) was used as the mobile phase at a flow-rate of 1.0 ml/min. The column temperature was maintained at 25°C. KA separated under these conditions was treated with 1 *M* zinc acetate (used as the post-column reagent), delivered at a flow-rate of 1.0 ml/min by a Shimadzu Model PRR-2A pump unit. KA–Zn^{2+} was measured at an excitation wavelength of 344 nm (10-nm bandpass) and at an emission wavelength of 398 nm (10-nm bandpass). The HPLC system was interfaced with a Shimadzu Chromatopac C-R3A for data processing.

Etraction of KA from urine

The preparative method for HPLC injection from urine is outlined in Fig. 1. KA in urine at acidic conditions (final concentration of hydrogen chloride, 0.1

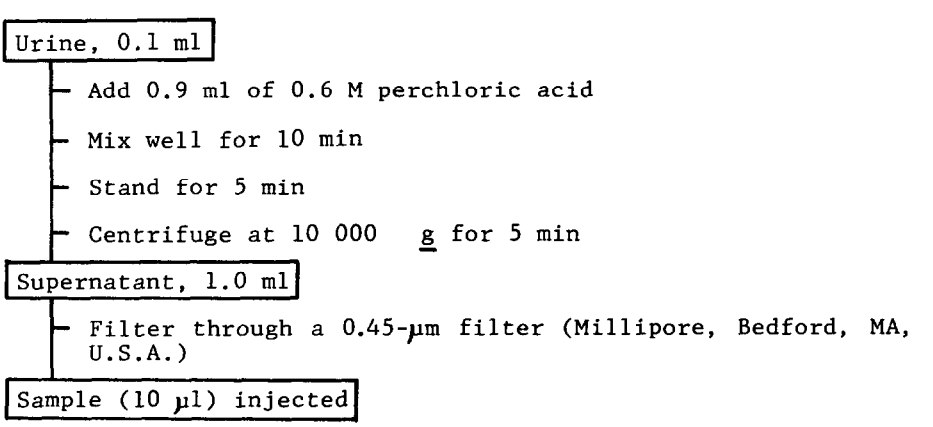


Fig. 1. Procedure for extraction of KA from urine.

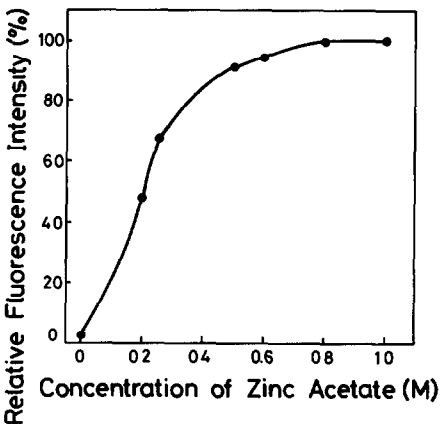


Fig. 2. Effect of the concentration of zinc acetate as the post-column reagent on the fluorescence intensity of KA. The values are means of two separate experiments.

M) was stable at least one month when the urine was stored at −25 °C.

The standard KA (21 mg) was dissolved in 100 ml of 3 m*M* sodium hydroxide and then diluted 100-fold with 0.6 *M* perchloric acid. Under these conditions, KA was stable for at least five days at 4 °C. However, KA degraded rapidly after four days when it was diluted 100-fold with water. The concentration of KA was calculated with the molar absorptivity of 42 500 at 244 nm in 0.6 *M* perchloric acid.

Effect of concentration of zinc acetate as the post-column reagent on the fluorescence intensity of KA

Fig. 2 shows the relationship between the concentrations of zinc acetate as the post-column reagent and the fluorescence intensity of KA. The highest fluorescence intensity was obtained with 0.8–1 *M* zinc acetate. When 1 *M* zinc acetate was delivered at a flow-rate of 1.0 ml/min, the fluorescence intensity of KA increased ca. 50-fold.

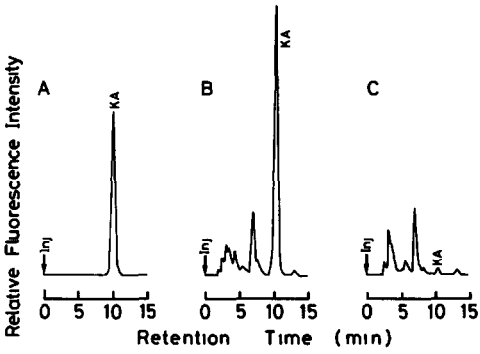
RESULTS AND DISCUSSION

Calibration curve and limit of detection

The calibration curve for KA was linear in the range 0.2–300 pmol per injection, with a correlation coefficient of 0.999. The linear regression equation was: KA (pmol) = (0.0001251 ± 0.0000006) × (integrated peak area) + (1.853 ± 1.728). The amount of KA was calculated using the following equation: KA (pmol) = integrated peak area/8000. The relative standard deviation of the peak area of KA (10 pmol per injection) was less than 1%. The detection limit was 0.2 pmol (41.8 pg) at a signal-to-noise ratio of 5:1.

Recovery of KA from urine

A 10-μl aliquot of 1 m*M* KA (10 nmol) was added to urine and processed as described in Fig. 1 (but 0.9 ml of 0.6 *M* perchloric acid was reduced to 0.89 ml). The recovery of KA from human urine was 99.8 ± 1.2% (mean ± S.D., $n=5$).



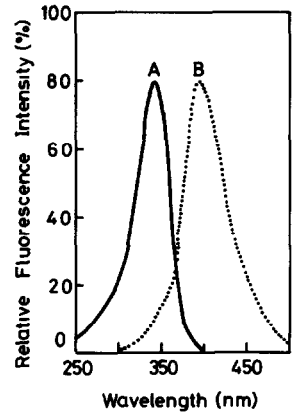


Fig. 3. Chromatograms of KA. Conditions: column, 5-ODS-H (150 mm×4.6 mm I.D.); mobile phase, 10 m*M* sodium acetate–acetic acid buffer (pH 4.5)–acetonitrile (95:5, v/v); flow-rate, 1.0 ml/min; post-column reagent, 1 *M* zinc acetate at a flow-rate of 1.0 ml/min; excitation wavelength, 344 nm; emission wavelength, 398 nm; column temperature, 25°C. (A) Chromatogram of standard KA (68 pmol); Shimadzu Chromatopac C-R3A, attenuation 5. (B) Chromatogram of an extract of human urine; sample size, 10 μl; KA, 27.2 pmol; Shimadzu Chromatopac C-R3A, attenuation 3. (C) Chromatogram of an extract of the same human urine sample as in (B) but with water instead of 1 *M* zinc acetate; Shimadzu Chromatopac C-R3A, attenuation 3.

Fig. 4. Excitation (A) and emission (B) spectra of KA peak by stop-flow and scanning mode. The excitation spectrum was obtained with an emission wavelength of 398 nm and the emission spectrum was obtained with an excitation wavelength of 344 nm.

TABLE I

URINARY EXCRETION OF KA IN JAPANESE COLLEGE WOMEN

Urine was collected under acidic conditions for 24 h and stored at −25°C until analysis.

Subject	KA (μmol in daily urine)	Subject	KA (μmol in daily urine)
1	5.54	14	8.61
2	5.71	15	8.87
3	5.86	16	8.93
4	5.87	17	9.01
5	6.58	18	9.34
6	6.89	19	9.85
7	6.92	20	10.07
8	7.03	21	10.21
9	7.54	22	10.30
10	7.98	23	10.78
11	8.10	24	11.77
12	8.14	25	12.00
13	8.43	Mean ± S.D.	8.41 ± 1.85

A typical chromatogram of the reference KA is shown in Fig. 3A. KA is eluted at ca. 10 min and the total HPLC analysis time is ca. 15 min. Fig. 4 shows the excitation and emission spectra of KA obtained on-line.

Human urinary excretion of KA

The chromatogram of an extract of a urine sample from a Japanese female student is shown in Fig. 3B. KA in the sample was characterized on the basis of its retention time and the entire excitation and emission spectra between 250 and 450 nm. Furthermore, KA was also characterized on the basis of the observation that the fluorescence intensity was extremely reduced when water was used instead of 1 *M* zinc acetate, as shown in Fig. 3C. The KA analogue, xanthurenic acid, was eluted at ca. 8.7 min (detected by the UV method); however, it was not detected fluorimetrically. The mean KA excretion in the female subjects studied ($n=25$, 20–21 years old) was 8.41 ± 1.85 μmol in daily urine, as shown in Table I. This value was about the same as the value reported by Iinuma et al. [15]; however, it is slightly lower than the values (women, 13.3 ± 4.0 μmol in daily urine; men, 13.2 ± 1.8 μmol in daily urine) reported by Price et al. [17]. This may be due to a difference of protein (tryptophan) intake between Japanese and Americans.

This method is straightforward and highly reproducible. The author believes this method is most useful for determination of KA in biological fluids.

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