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High-performance liquid chromatographic determination of 3-hydroxykynurenine with fluorimetric detection; comparison of preovulatory phase and postovulatory phase urinary excretion

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ABSTRACT

A high-performance liquid chromatographic assay for 3-hydroxykynurenine in human urine is described. A fluorescent derivative of 3-hydroxykynurenine was prepared, based on the reaction of the compound with *p*-toluenesulphonyl chloride in a basic medium. The analytical method for the measurement of the fluorescent compound employed a Tosoh ODS 80 column eluted with 10 m*M* potassium dihydrogenphosphate (pH 4.5) and acetonitrile (3:2, v/v) and detection at an excitation wavelength of 375 nm (10 nm bandpass) and an emission wavelength of 455 nm (10 nm bandpass). The column temperature was maintained at 25°C. The detection limit was 3 pmol (673 pg) at a signal-to-noise ratio of 5:1. The fluorescent derivative of 3-hydroxykynurenine was eluted at *ca.* 12.5 min. The technique was applied to the analysis of human urine. The total analysis time was *ca.* 15 min.

INTRODUCTION

The investigation of tryptophan–niacin metabolism is a subject of considerable clinical, biochemical and nutritional importance. Urinary excretion of the intermediates of the tryptophan–niacin pathway is the most useful index of the systemic metabolism of tryptophan–niacin. To this end, we have reported high-performance liquid chromatographic (HPLC) methods for the estimation of anthranilic acid [1], 3-hydroxyanthranilic acid [1], kynurenic acid [2], nicotinamide [3], N^1-methylnicotinamide [4], N^1-methyl-2-pyridone-5-carboxamide [5], N^1-methyl-4-pyridone-3-carboxamide [6], nicotinic acid [7], nicotinuric acid [7] and nicotinamide N-oxide [8].

The effect of vitamin B_6 deficiency on urinary excretion of the metabolites of the tryptophan–niacin pathway is well known [9–26]. In the main pathway of tryptophan–niacin, vitamin B_6 is involved in the formation of 3-hydroxyanthranilic acid from 3-hydroxykynurenine (3-HK). Therefore, the urinary excretion ratio of 3-HK to 3-hydroxyanthranilic acid would be a valuable index of the status of vitamin B_6. In fact, O'Brien and Jensen [27] and Heeley [28] suggested

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that an increase in the ratio of 3-HK to 3-hydroxyanthranilic acid would be a reliable indication of vitamin B_6 deficiency. The determination of urinary excretion of 3-HK was reported by Watanabe *et al.* [29], in which 3-HK was derivatized by the reaction with *p*-toluenesulphonyl chloride in a basic medium to yield a fluorescent compound, which was separated by thin-layer chromatography (TLC). HPLC is more convenient and powerful than TLC, and 3-HK was quantified by HPLC with electrochemical detection [30,31]. However, these methods cannot be applied to the determination of 3-HK in urine.

We have therefore developed an isocratic reversed-phase HPLC assay with fluorimetric detection of the derivative of 3-HK. Furthermore, in the present report, urinary excretion of 3-HK in preovulatory phase (follicular phase) and postovulatory phase (luteal phase) was compared, since the excretion of 3-HK was found to be significantly higher in the prepuberty phase than in the sexual maturity phase [32].

EXPERIMENTAL

Chemicals

p-Toluenesulphonyl chloride was purchased from Wako (Osaka, Japan). 3-HK was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All the other chemicals used were of the highest commercial grade available.

Derivatization of 3-HK

3-HK was derivatized as described by Watanabe *et al.* [29], except that each volume was reduced and the purification steps with diethyl ether were omitted because they did not offer any advantage. The concentration of 3-HK was calculated using the molar absorptivity of 7140 M^{-1} cm^{-1} at 269 nm in water. Then 800 μl of 0.5% *p*-toluenesulphonyl chloride (in acetone) and 200 μl of 5% $NaHCO_3$ were added to 600 μl of diluted urine in a microtube with a sealed cap. After 10 min at room temperature, the microtube was centrifuged at 8000 *g* for 3 min. The resulting supernatant was retained and filtered through a 0.45-μm filter (Millipore, Bedford, MA, USA) and the filtrate (20 μl) was directly injected into the HPLC system.

HPLC conditions

HPLC was carried out with a Shimadzu LC-4A equipment and a Shimadzu RF-540 spectrofluorimeter equipped with an LC flow-cell unit (Shimadzu, Kyoto, Japan), a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA), and a Tosoh ODS 80 column (150 mm × 4.6 mm I.D., particle size 5 μm, Tosoh, Tokyo, Japan). A degassed solution of 10 mM KH_2PO_4 (pH 4.5) and acetonitrile (3.2, 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. The derivative of 3-HK was measured at an excitation wavelength of 375 nm (10 nm bandpass) and at an

emission wavelength of 455 nm (10 nm bandpass). The HPLC system was interfaced with a Shimadzu Chromatopac C-R3A for data processing.

Urine collection

Sexually mature women aged 21–25 years old took part in the experiment. Urine samples (24 h) from the preovulation phase (the 9th day of the menstrual cycle) and the postovulation phase (the 21st day of the menstrual cycle) were collected in bottles containing a few milliliters of 1 *M* HCl and stored at −25°C until analysis.

RESULTS AND DISCUSSION

Stability of the fluorescent compound of 3-HK

The fluorescent compound was stable: even when the reaction mixture was kept at room temperature for two months, no measurable loss of the fluorescent compound of 3-HK was observed.

Calibration curve and limit of detection

The calibration curve for the fluorescent compound of 3-HK was linear in the range 3–138 pmol per injection, with a correlation coefficient of 0.999. The linear regression equation was: 3-HK (pmol) = $(2.54 \cdot 10^{-3} \pm 3.78 \cdot 10^{-5}) \times$ (integrated peak area) + (0.57 ± 1.62). The amount of 3-HK was calculated using the following equation: 3-HK (pmol) = integrated peak area/394. The coefficient of variation (C.V.) of 3-HK (13.75 pmol per injection) was less than 1%. The detection limit was 3 pmol (673 pg) at a signal-to-noise ratio of 5:1.

Validation of the method

To check the validity of the method, a pooled urine sample was measured five times and the resulting C.V. was 3.9% as shown in Table I (in this case, the reaction mixture contained 0.8 ml of 0.5% *p*-toluenesulphonyl chloride, 0.2 ml of 5% $NaHCO_3$, 0.3 ml of the pooled urine sample and 0.3 ml of water). In order to determine the recovery of 3-HK from human urine, 0.3 ml of 11 μM 3-HK (3.3 nmol) was added to the mixture of 0.8 ml of 0.5% *p*-toluenesulphonyl chloride, 0.2 ml of 5% $NaHCO_3$ and 0.3 ml of the pooled urine sample. The recovery was 97%, as shown in Table I.

A typical chromatogram of the reference 3-HK derivative is shown in Fig. 1A. The 3-HK derivative eluted at *ca.* 12.5 min. Fig. 2 shows the excitation and emission spectra of the 3-HK derivative obtained on-line.

Application of the method

The chromatogram of a derivatized urine sample is shown in Fig. 1B. The 3-HK derivative in the sample was characterized on the basis of its retention time and the entire excitation and emission spectra between 320 and 500 nm. The peak

TABLE I

PRECISION AND RECOVERY OF 3-HYDROXYKYNURENINE FROM URINE

Added (nmol)	Found (mean ± S.D., n = 5) (nmol)	C.V. (%)	Recovery (%)
0	0.77 ± 0.03	3.9	–
3.3	3.96 ± 0.20	5.1	97[a]

[a] (3.96 − 0.77)/3.3 × 100 = 97.

was further characterized by comparison of chromatograms in the presence and in the absence of *p*-toluenesulphonyl chloride, as shown in Fig. 1B and C. The total HPLC analysis time was *ca.* 15 min.

The present derivatization method is straightforward, and the 3-HK derivative is very stable. The 3-HK peak can be easily identified because no peak is observed at *ca.* 12.5 min when *p*-toluenesulphonyl chloride is omitted from the reaction mixture.

It was reported that the spontaneous excretion of 3-HK is higher in the prepuberty phase than in the sexual maturity phase, and that of 3-hydroxyanthranilic acid is higher in the postovulatory phase than in the preovulatory phase of the menstrual cycle [32]. Therefore, the urinary excretion of 3-HK in the pre- and

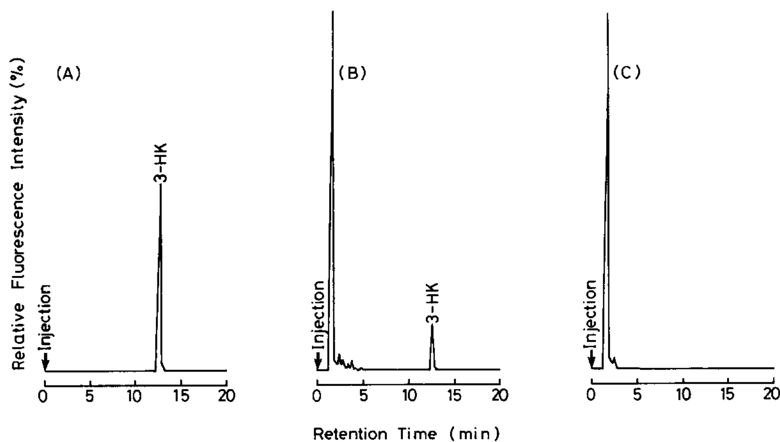

Fig. 1. Chromatograms of the 3-hydroxykynurenine derivative. Conditions: column, Tosoh ODS 80 (150 mm × 4.6 mm I.D.); mobile phase, 10 mM KH_2PO_4 (pH 4.5)–acetonitrile (3:2, v/v); flow-rate, 1.0 ml/min; excitation wavelength, 375 nm; emission wavelength, 455 nm; column temperature, 25°C. (A) Chromatogram of standard 3-hydroxykynurenine (750 pmol); Shimadzu Chromatopac C-R3A, attenuation 5. (B) Chromatogram of a urine sample after derivatization; sample size, 20 μl; 3-hydroxykynurenine, 45 pmol; Shimadzu Chromatopac C-R3A, attenuation, 3. (C) Chromatogram of the same urine sample as in (B) but without *p*-toluenesulphonyl chloride; Shimadzu Chromatopac C-R3A, attenuation, 3.

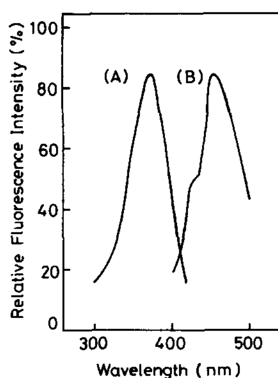

Fig. 2. Excitation (A) and emission (B) spectra of the 3-hydroxykynurenine derivative peak by stop-flow and scanning mode. The excitation spectrum was obtained with an emission wavelength of 455 nm and the emission spectrum was obtained with an excitation wavelength of 375 nm.

TABLE II

COMPARISON OF THE URINARY EXCRETION OF 3-HYDROXYKYNURENINE BETWEEN PREOVULATORY PHASE AND POSTOVULATORY PHASE

Subject No.	3-Hydroxykynurenine (μmol per daily urine)	
	Preovulatory	Postovulatory
1	1.1	7.3
2	1.3	7.4
3	0.4	7.2
4	3.2	4.8
5	2.9	2.0
6	4.2	5.6
Mean ± S.D.	2.2 ± 1.5	5.7 ± 2.1

TABLE III

REPORTED VALUES OF URINARY EXCRETION OF 3-HYDROXYKYNURENINE

Values are mean ± S.D.

Subject	Age (years)	3-Hydroxykynurenine (μmol per daily urine)	*n*	Reference
Prepuberty female	8–12	56.5 ± 17.8	15	32
Sexually mature female	23–40	4.9 ± 2.4	22	32
Menopausal female	50–65	14.5 ± 4.3	23	32
Adult male	24–41	4.1 ± 3.0	30	29
Sexually mature female	22–42	4.8 ± 1.4	4	29
Sexually mature female	20–26	6.8 ± 7.2	6	23

postovulatory phases was compared. As shown in Table II, the urinary excretion of 3-HK was higher in the postovulatory phase than in the preovulatory phase. A possible explanation of this difference may be that the formation of 3-HK from kynurenine (catalysed by kynurenine 3-hydroxylase, which is found in the outer membrane of the mitochondria), where NADPH and FAD are involved [33], varies with the menstrual cycle. The reported values for 3-HK are summarized in Table III. The value obtained by the present method for sexually mature females was almost the same as the reported values.

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