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Short communication

Determination of 8-methyl ether of xanthurenic acid in human urine by high-performance liquid chromatography

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Abstract

We developed a simple and sensitive assay for the urinary 8-methyl ether of xanthurenic acid (XA-OMe) by high-performance liquid chromatography with fluorescence detection (excitation at 340 nm; emission at 450 nm). Urine samples were diluted with 0.03 *M* potassium phosphate buffer (pH 6.0) and applied to an octadecylsilane-bonded column (Nucleosil $5C_{18}$, 150 × 4 mm I.D.). The mobile phase used was a mixture of this same buffer and acetonitrile (1000:140, v/v). Both direct injection of urine and solvent extraction prior to HPLC were tested and showed a good correlation and sensitivity, although the peak of XA-OMe was occasionaly less distinguishable from close peaks in urine from normal controls by the direct injection method. The quantification limit was $5 \cdot 10^{-14}$ mol which was sensitive enough to detect XA-OMe in urine from normal subjects. The method was applied to samples from patients with a deficiency in tryptophan catabolism, xanthurenic acid/3-hydroxykynurenine-uria and showed a striking elevation in urinary XA-OMe excretion.

1. Introduction

The 8-methyl ether of xanthurenic acid (XA-OMe) is a metabolite of tryptophan catabolism and suspected of being carcinogenic. Fujinaga [1] reported high XA-OMe levels in urine from patients with bladder cancer as measured by ion exchange-extraction and thin-layer chromatography-densitometry. To our knowledge, this is the only report dealing with an analytical method for the determination of urinary XA-OMe. However this method is fairly time-consuming and thus may be inconvenient for routine screening. In this paper we report a simple and sensitive high-performance liquid chromatographic (HPLC) technique for the determination of XA-OMe in urine and results for urine samples from patients with a disorder of tryptophan catabolism, xanthurenic acid/3-hydroxykynurenine-uria (XA/HKyn-uria) are described.

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2. Experimental

2.1. Apparatus

A Model 655A-11 solvent delivery system, Model F-1000 fluorescence spectrophotometer and a Model 655A-52 column oven, all from Hitachi (Tokyo, Japan), were used. A Model 851-AS autoinjector (Jasco, Kyoto, Japan) and Model KF-21 degasser (Showadenko, Tokyo, Japan) were connected in series. A stainless steel column (150 × 4 mm I.D.) packed with Nucleosil $5C_{18}$ (Macherey-Nagel, Germany) was used.

2.2. Reagents and chemicals

XA-OMe was synthesized in our laboratory by the method of Furst and Olsen [2], and Price and Dodge [3] (m.p. 240°C, elemental analysis, calculated: H 4.14%, C 60.28%, N 6.39%; found: H 4.09%, C 60.01%, N 6.39%). Acetonitrile was HPLC grade from Nacalai Tesque (Kyoto, Japan) and the other reagents were reagent grade.

2.3. Chromatographic conditions

The mobile phase was prepared by mixing 0.03 *M* potassium phosphate monobasic (KH_2PO_4, pH 6.0) with acetonitrile (1000:140, v/v) containing 0.1 m*M* Na_2EDTA, followed by filtration through a 0.2-μm membrane filter (Advantec Toyo, Tokyo, Japan). The flow-rate was 0.7 ml/min, and the column temperature was 40°C. Five microliters of the test samples (prepared as described below) were injected onto the column and the fluorescence intensity of the peaks was monitored using an excitation wavelength of 340 nm and an emission wavelength of 450 nm.

2.4. Preparation of the test samples

Direct dilution method

Human urine samples were collected in test tubes and kept frozen at −25°C until analysis. The urine samples were thawed in a water bath and diluted ten- to twenty-fold with 0.03 *M* KH_2PO_4 (pH 6.0) and filtered through a 0.45-μm membrane filter (Advantec Toyo).

Extraction method

A 1.5-ml aliquot of urine sample was pipetted into a 10-ml glass tube with a cap, to which were added 0.6 g of sodium chloride, 0.5 ml of water, 0.5 ml of 0.4 *M* hydrochloric acid and 3.5 ml of ethyl acetate. The mixture was shaken vigorously on a reciprocal shaker for 10 min and centrifuged at 1200 *g* for 5 min. The separated organic layer was transferred with a Pasteur pipet to a 10-ml roundbottom flask. The extraction was repeated twice each with 3.5 ml of ethyl acetate. The combined organic layers were evaporated to dryness on a water bath at 40°C under reduced pressure. The residue was dissolved in 5.0 or 10.0 ml of 0.03 *M* KH_2PO_4 (pH 6.0) and filtered through a 0.45-μm membrane filter.

2.5 Identification of peaks

Initial identification of the chromatographic peaks in the urine samples was made on the basis of their retention times and co-chromatography of the reference compounds. Further proof of the peak identity was obtained by HPLC with fast-atom bombardment mass spectrometry [4].

3. Results and discussion

The maximum fluorescence intensity of XA-OMe was obtained at 340 nm (excitation) and 450 nm (emission) (Fig. 1). The excitation and emission wavelengths exhibiting maximum fluorescence were the same for different pH values (pH 3–6), but at pH 6.0 the fluorescence intensity at emission was 2.65-fold higher than at pH 3.0 (Fig. 2).

Progressive increase in the concentration of acetonitrile in the mobile phase slightly enhanced the fluorescence intensity of XA-OMe but shortened the retention times. Excessive shortening of the retention times caused a difficulty in the chromatographic separation between the XA-OMe peak and the other peaks in some urine samples. Thus a mobile phase of 0.03 *M*

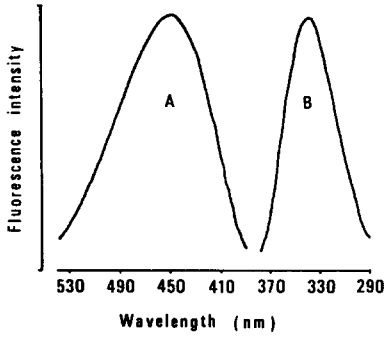

Fig. 1. Fluorescence spectra of XA-OMe in 0.03 *M* KH_2PO_4 (pH 6.0)–acetonitrile (1000:140, v/v) solution. Emission spectrum (A) was obtained with excitation at 340 nm and excitation spectrum (B) with emission at 450 nm.

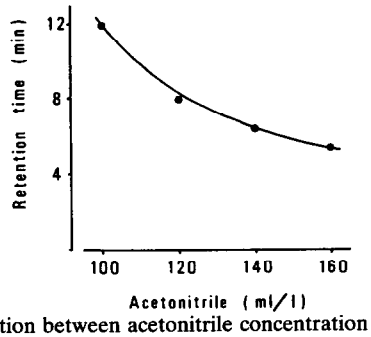

Fig. 3. Relation between acetonitrile concentration in mobile phase with the retention time of XA-OMe. Acetonitrile was mixed with 0.03 *M* KH_2PO_4 (pH 6.0) in ratio 100–160:1000 (v/v).

KH_2PO_4–acetonitrile (1000:140, v/v) was employed (Fig. 3).

The calibration graphs of the peak area versus the amount of XA-OMe were linear in the range of 0.002–1.0 μg/ml (0.01–5 ng) of XA-OMe. The detection limit was calculated to be $2 \cdot 10^{-15}$ mol in a 5-μl injection volume at a signal-to-noise ratio of 5 and the lower limit of quantification was calculated to be $5 \cdot 10^{-14}$ mol (21 ng/ml in urine) at a C.V. of 10%.

The direct dilution method gave a suitable separation of XA-OMe in urine from patients but not in urine from normal subjects. The method using extraction of XA-OMe with ethyl acetate in acidic medium containing hydrochloric acid was found to give a satisfactory separation of XA-OMe in all cases.

Typical chromatograms of urine samples (Fig. 4) show that a normal adult urine sample processed with the direct dilution method contains

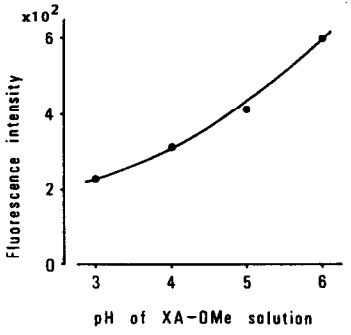

Fig. 2. pH dependence of the fluorescence intensity of XA-OMe. XA-OMe was dissolved in 0.03 *M* KH_2PO_4–acetonitrile (1000:140, v/v) with pH 3–6 (2 μg/ml). The fluorescence intensity was measured at 450 nm with excitation at 340 nm.

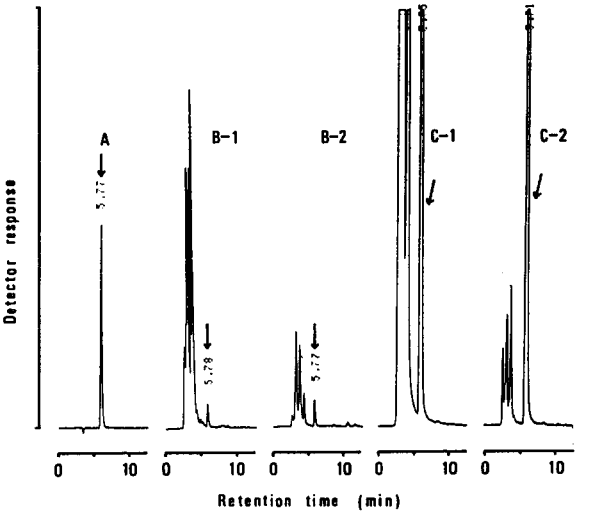

Fig. 4. HPLC chromatograms for urinary XA-OMe. (A) XA-OMe standard (0.1022 μg/ml), (B) normal adult urine processed by the direct dilution method (B-1, 0.0107 μg/ml, diluted ten-fold) and by the extraction method (B-2, 0.0108 μg/ml, the extracts from 0.5 ml of urine were dissolved in 5.0 ml of 0.03 *M* KH_2PO_4 (pH 6.0)), (C) urine from case 1 with XA/HKyn-uria by the direct dilution method (C-1, 1.12 μg/ml, diluted twenty-fold) and by the extraction method (C-2, 1.10 μg/ml, the extracts from 0.5 ml of urine were dissolved in 10.0 ml of 0.03 *M* KH_2PO_4 (pH 6.0)). Case 1: see Table 1. Chromatographic conditions: see text.

Table 1
Recovery of XA-OMe from normal urine

XA-OMe added to normal urine (ng)	Direct dilution method		Extraction method	
	Found (ng)	Recovery (%)	Found (ng)	Recovery (%)
50	46.31	92.61	49.70	99.41
	46.98	93.96	53.82	107.6
100	98.66	98.66	106.3	106.3
	98.68	98.68	106.6	106.6
200	217.0	108.5	214.6	107.3
	201.6	100.8	206.0	103.0
400	398.3	99.58	391.5	97.87
	401.4	100.3	394.5	98.62
Average		99.85 ± 4.82		103.3 ± 4.18

some unknown peaks before and close to the XA-OMe peak. Pretreatment of urine samples from a normal adult and a case of XA/HKyn-uria with the extraction method reduces the prominent chromatographic peaks eluted prior to XA-OMe in the void volume to such an extent that the XA-OMe peak is clearly distinguishable. Thus the extraction method will be preferable for good separation of XA-OMe.

Recoveries of XA-OMe with the direct dilution method and the extraction method were 92–108% and 97–107%, respectively (Table 1). Analytical results obtained by the direct dilution method were compared with those obtained by extraction of the same urine samples (Fig. 5).

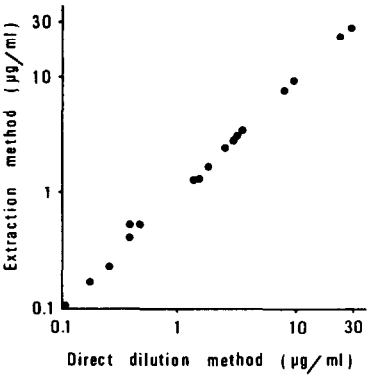

Fig. 5. Correlation between the urinary XA-OMe values obtained by the direct dilution method and the extraction method.

Table 2
Urinary excretion of the metabolites of tryptophan in patients and normal controls (μg/mg cre)

Metabolite	Case 1 (female, 1.9 years)	Case 2 (female, 8 months)	Case 3 (male, 8 months)	Controls		
				Mean	Range	*n*
XA	111	40.6	148	2.5	0.0–5.0	12
HKyn	618	488	553	3.6	0.4–10.4	12
XA-OMe	30.5	17.1	38.2	0.234	0.106–0.422	10

XA = xanthurenic acid, HKyn = 3-hydroxykynurenine, XA-OMe = 8-methyl ether of xanthurenic acid, cre = creatinine. Case 1,2,3: patients with xanthurenic acid/3-hydroxykynurenine-uria; controls: normal infants [XA, HKyn: 4 females and 8 males, 1.1–4.0 (average 2.3) years old; XA-OMe: 3 females and 7 males, 1.25–4.0 (average 2.5) years old]. XA and HKyn were determined by ion-pair HPLC with electrochemical detection [8].

The results agreed closely; the regression equation was $y = 0.9797x + 0.0164$ ($r = 0.998$).

XA-OMe excretion was much enhanced in three patients with XA/HKyn-uria compared to that in normal infants (Table 2). Since XA-OMe is derived from XA in animal tissues [5–7], enhanced excretion of XA in the above patients may be responsible for the increase in urinary XA-OMe. XA-OMe is considered to be a possible bladder carcinogen in mice and its enhanced excretion was observed in patients with bladder cancer [1]. Further studies are needed to clarify whether infants with an increased excretion of XA-OMe due to an inborn error in the tryptophan metabolism, such as XA/HKyn-uria, stand a high risk of developing bladder cancer.

The presented analytical method for determination of urinary XA-OMe by HPLC is very simple and highly sensitive. Thus we consider it to be applicable to routine clinical testing or study of tryptophan metabolism.

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