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Technical note

Determination of the 8-methyl ether of xanthurenic acid in human serum by high-performance liquid chromatography with fluorescence detection

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Abstract

We developed a simple and sensitive assay for the 8-methyl ether of xanthurenic acid in serum by high-performance liquid chromatography with fluorescence detection (excitation at 340 nm, emission at 450 nm). The compound under study in serum samples was extracted with Sep-Pak C_{18} cartridge and the extract was applied to an octadecylsilane-bonded column (Nucleosil $5C_{18}$, 150×4 mm I.D.). The mobile phase used was a mixture of 0.05 *M* sodium acetate buffer (pH 6.0), containing 5 m*M* sodium 1-octanesulfonate and 0.1 m*M* Na_2EDTA, and acetonitrile (93:7, v/v). The 8-methyl ether of xanthurenic acid from serum samples was sufficiently separated to be clearly distinguishable. The quantification limit was 2×10^{-14} mol, which was sensitive enough to detect 8-methyl ether of xanthurenic acid in serum from normal subjects. The method was applied to samples from patients with deficiency in tryptophan metabolism, xanthurenic acid/3-hydroxy kynurenineuria and showed a striking elevation in serum 8-methyl ether of xanthurenic acid.

Keywords: Xanthurenic acid, 8-methyl ether; Tryptophan

1. Introduction

The 8-methyl ether of xanthurenic acid (XA-OMe) is a metabolite of tryptophan catabolism which is suspected of being carcinogenic [1], and its enhanced urinary excretion was observed in patients with bladder cancer [2]. We reported a simple and sensitive HPLC technique for the determination of urinary XA-OMe and enhanced excretion of XA-OMe in urine samples from patients with xanthurenic acid/3-hydroxylkynurenineuria (XA/HKyn-uria) [3] due to inborn error of tryptophan metabolism. XA/HKyn-uria patients have a reduced kynureninase activity resulting from an inability of the apoenzyme to combine normally with the coenzyme (pyridoxal phosphate) [4]. Urinary excretion, in general, reflects the blood levels. Therefore the amounts of XA-OMe in blood are supposed to be high in those patients, but we could not find in the literature any technique reported for XA-OMe determination in blood. In this

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paper we report a simple and sensitive HPLC method for the quantification of XA-OMe in serum and the results for serum samples from normal adults and from patients with XA/HKyn-uria.

2. Experimental

2.1. Apparatus

A Model 655A-11 solvent delivery system, Model F-1000 fluorescence spectrophotometer and 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 as previously reported (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%) [3]. Acetonitrile was HPLC grade from Nacalai Tesque (Kyoto, Japan) and the other reagents were reagent grade. Sep-Pak C_{18} cartridges (part No. 51910) were purchased from Waters (Milford, MA, USA). A stock solution of 100 μg/ml of XA-OMe in 0.01 *M* NaOH was prepared. The solution was stable for 6 months at 4°C. A working reference solution of 1 ng/ml of XA-OMe was prepared daily with the HPLC mobile phase.

2.3. Serum samples

Serum was separated from venous blood by centrifugation at 1000 *g* for 10 min at 4°C and was stored at −25°C until the analysis.

2.4. Preparation of the test samples

A serum sample (0.5 ml) was pipetted into a small test tube and was diluted with 5 ml of 0.05 *M* sodium acetate buffer (pH 6.0) and mixed well on the Vortex-type mixer.

A 5-ml volume of diluted serum was applied onto Sep-Pak C_{18} cartridge, activated beforehand by successive elution with 20 ml each of methanol, water and 0.05 *M* sodium acetate buffer (pH 6.0). Methanol (10 ml) was added to the cartridge after washing with 10 ml water. The methanolic effluent was collected into a 10-ml round-shaped flask and was evaporated to dryness at 40°C under reduced pressure. The residue was dissolved in 1 ml of HPLC mobile phase and filtered through a 0.2-μm membrane filter (Advantec Toyo, Tokyo, Japan).

2.5. Chromatographic conditions

The mobile phase was prepared by mixing 0.05 *M* sodium acetate buffer (pH 6.0), containing 5 m*M* sodium 1-octanesulfonate and 0.1 m*M* Na_2EDTA, with acetonitrile (93:7, v/v) followed by filtration through a membrane filter, pore size 0.2 μm. The flow-rate was 0.5 ml/min, and the column temperature was 40°C. A 100-μl aliquot of the test samples was injected onto the column and the fluorescence intensity of peaks was monitored by using an excitation wavelength of 340 nm and an emission wavelength of 450 nm as previously described [3].

3. Results and discussion

XA-OMe in serum was sufficiently retained on Sep-Pak C_{18} cartridges. It was thus separated from serum proteins, in 0.05 *M* sodium acetate buffer (pH 6.0) medium and was completely eluted with 10 ml methanol. XA-OMe eluted in the methanolic fraction and was sufficiently separated by HPLC, as shown in Fig. 1. The elution time of XA-OMe was 5.4 min.

The HPLC retention times of the major tryptophan metabolites and XA-OMe are given in Table 1. Though kynurenine eluted at 5.46 min, shortly after XA-OMe at 5.37 min, it had no fluorescence under the analytical conditions used and did not interfere with the assay of XA-OMe. None of the other metabolites interfered with the determination of XA-OMe because of their different retention times and/or their negligible fluorescence intensity at the excitation wavelength of 340 nm and at emission wavelength of 450 nm.

The calibration graphs of the peak versus the

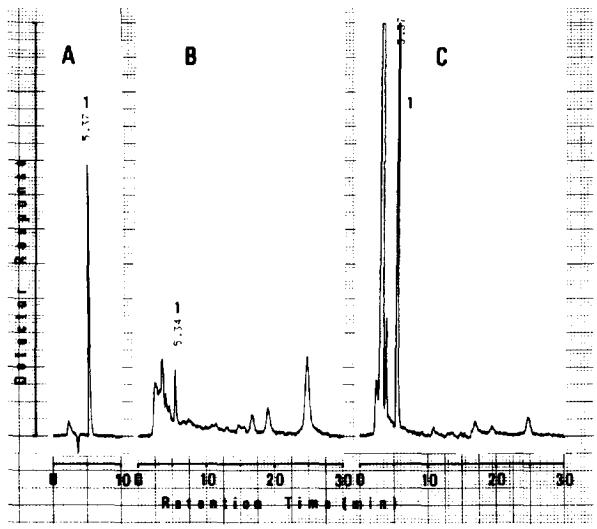

Fig. 1. HPLC chromatograms for serum XA-OMe. (A) XA-OMe standard (1.15 ng/ml), (B) normal adult serum processed by the procedure described in the text (0.250 ng/ml), (C) serum from case 2 with XA/HKyn-uria (2.94 ng/ml). 0.1 ml serum was diluted with 5 ml 0.05 *M* sodium acetate buffer (pH 6.0) and a 5 ml aliquot was subjected to Sep-Pak extraction. Peak 1 = XA-OMe. Unmarked are unidentified serum components.

amount of XA-OMe were linear in the range of 0.1–25 ng/ml (0.01–2.5 ng) of XA-OMe. The regression equation was:

$$\text{Peak area } (\mu A) = 100\,212 \cdot \text{XA-OMe (ng/ml)} - 2828 \quad (r = 0.99998)$$

The detection limit was calculated to be $6 \cdot 10^{-15}$ mol/100 μl injection volume at a signal-to-noise ratio of 5, and the lower limit of quantification was calculated to be $3.1 \cdot 10^{-14}$ mol (0.150 ng/ml in serum) with a C.V. of 10%.

The recoveries of 0.5, 1.0 and 5.0 ng of XA-OMe

Table 1
Retetion times of the major tryptophan metabolites and XA-OMe

Tryptophan metabolites	Retention time (min)	$\lambda_{max.Ex}/\lambda_{max.Em}$ (nm)[a]	Relative fluorescence intensity at λ_{Ex} 340 and λ_{Em} 450[b]
XA-OMe	5.37	340/450	100
Tryptophan	7.98	–[c]	0
Serotonin	42.50	305/335	0.002
5-HIAA	2.88	–	0
Kynurenine	5.46	–	0
HKyn	3.72	–	0
KA	3.26	292/365	0.020
QA	2.15	325/375	0.004
Quinaldic acid	4.22	320/430	0.023
Picolinic acid	2.61	–	0
HAA	2.75	330/405	5.13
AA	3.40	340/395	4.40

XA-OMe, 8-methylether of xanthurenic acid; 5-HIAA, 5-hydroxyindole acetic acid; HKyn, 3-hydroxykynurenine; KA, kynurenic acid; QA, quinolic acid; HAA, 3-hydroxy anthranilic acid; AA, anthranilic acid.

[a]Excitation (Ex) and emission (Em) wavelength at which the maximum fluorescence intensity in HPLC mobile phase was obtained.

[b]Relative fluorescence intensity of 1 μM solution in HPLC mobile phase.

[c]No fluorescence was observed in 1 μM solution in HPLC mobile phase.

Table 2
Levels of XA-OMe in serum from normal adults and patients with XA-HKyn-uria

Subject	Sex	Age (years)	XA-OMe (ng/ml)
Normal adult			
1	M	31	0.419
2	M	43	0.602
3	M	53	0.470
4	M	57	0.914
5	M	59	0.521
6	M	61	0.289
7	F	36	0.506
8	F	46	1.59
9	F	48	0.758
10	F	51	0.215
11	F	57	0.157
Mean±S.D.			0.5873±0.5805
Case 1	F	6	52.0
Case 2	F	5	30.0

from serum were 88.27±4.63%, 98.08±4.98% and 79.86±6.02% (mean±S.D., obtained from three determinations), respectively.

The XA-OMe level in serum samples from normal adults was 0.5873±0.5805 ng/ml ($n=11$), and that in serum from two patients with XA/HKyn-uria was much enhanced, 52.0 and 30.0 ng/l, respectively (Table 2). These results may be responsible for the increase in urinary XA-OMe in these patients [3].

Further studies are needed to clarify whether infants with increased urinary excretion of XA-OMe and with elevation of serum concentration due to an inborn error in the tryptophan metabolism, such as XA/HKyn-uria, stand at high risk of suffering from cancer. Since our HPLC method for serum XA-OMe is simple and sensitive enough to detect normal levels, we consider the method to be applicable to clinical trials in which serum XA-OMe is used as an index of evaluating the nutritional status of vitamin B_6 or the tryptophan metabolism.

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