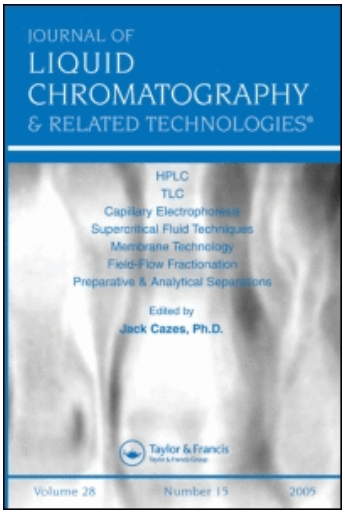

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR BIOTINIDASE ACTIVITY IN THE HUMAN URINE

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ABSTRACT

To date, no detectable biotinidase activity has been reported in the human urine. In the present study, a simple and convenient HPLC-fluorimetric assay method was applied to biotinidase activity measurement of urine samples. Biotinyl-6-aminoquinoline was utilized as the enzyme substrate, and the liberated product (6-aminoquinoline) was monitored with a fluorimetric detector. Biotinidase activity was assessed in urine specimens from: twenty-five patients with various renal disorders associated with proteinuria, and forty age- and sex-matched healthy control subjects. The examined samples from the patients with

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renal diseases exhibited biotinidase activity with the exception of six of the patients (enzyme activity, median: 49.9 $pmol \cdot min^{-1} \cdot ml^{-1}$ of urine; range: 0 - 2498 $pmol \cdot min^{-1} \cdot ml^{-1}$); specific activity, median: 19.4 $pmol \cdot min^{-1} \cdot mg^{-1}$ of protein; range: 0 - 176 $pmol \cdot min^{-1} \cdot mg^{-1}$) while none of the urine specimens from the control subjects showed detectable enzyme activity. Thus, this method was shown to be a successfully applicable for the quantitative analysis of biotinidase activity in the human urine.

INTRODUCTION

Biotinidase (EC 3.5.1.12) is an amidase which mainly hydrolyzes biotinyl-amido compounds, such as biocytin [1], biotinyl-4-aminobenzoate [2], and biotinyl-6-aminoquinoline (BAQ)[3]. Although detectable biotinidase activity has been reported in most mammalian tissues and body fluids [4], the unequivocal demonstration of enzyme activity in human body fluids is hitherto limited to serum [5] and breast milk [6]. Although the molecular weight of the serum enzyme protein is known (M_r: 76 kDa) [1, 5], no information on its primary structure is to date available. Furthermore, to date there is no data present in the literature on the urinary excretion of this enzyme. Despite the detection of its high specific activity in the kidneys of several animal species, no biotinidase activity has previously been reported in the urine [4].

Recently, a HPLC-fluorimetric method for biotinidase assay, with BAQ as substrate, was developed by us [7] and applied to the enzyme determination in the cerebrospinal fluid [8]. Using a similar method the biotinidase activity in the urine of healthy subjects and patients with various renal diseases associated with proteinuria was investigated.

MATERIALS AND METHODS

Chemicals: BAQ was purchased from Sigma (St. Louis, MO, USA). 6-aminoquinoline (AQ) was obtained from Aldrich (Milwaukee, USA); 2-mercaptoethanol was from Wako (Osaka, Japan).

Urine specimens: A total of 65 subjects were involved in the study. Urine samples from 25 patients [10 male, 15 female, age (mean ± SD) 14.8 ± 4.8 years] with renal disorders (diagnoses are shown in Table I) and proteinuria in excess of 150 mg/24 h were collected. The patients were on a normal diet, presented no evidence for associated liver disease, and received no drugs known to interfere with biotin metabolism.

Urine samples from forty control subjects (17 male, 23 female, age: 15.1 ± 4.0 years) were also obtained. A 0.2 ml volume of fresh urine from each sample was stored at - 80 °C until the date of assay; before the assay, samples were thawed and filtered (Ekicrodisc 13, pore size: 0.2 μm, Gelman Sciences Japan, Ltd, Tokyo, Japan).

Biotinidase assay : Biotinidase activities were determined by a HPLC fluorimetric method [7] using BAQ as substrate. BAQ was dissolved at 44 μM (16.3 mg/l) in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM (452 mg/l) Na_4 EDTA and 10 mM (781 mg/l) 2-mercaptoethanol. Substrate containing 90 μl of the reaction buffer was mixed with 10 μl of enzyme solution. Thus, the reaction mixture (100 μl) contained 0.001468 mg (3.96 nmol) of BAQ, 0.04 mg of EDTA and 0.07 mg of 2-mercaptoethanol. The reaction proceeded for an appropriate time at 37 °C, and was stopped by adding 200 μl of methanol; the reaction mixture was diluted three-fold with methanol in order to precipitate the enzyme

TABLE I

Biotinidase activity in urine samples from patients with renal disease *

Sample no.	Diagnosis	Protein content	Activity	Specific activity
		$mg \cdot ml^{-1}$	$pmol \cdot min^{-1} \cdot ml^{-1}$	$pmol \cdot min^{-1} \cdot mg^{-1}$
1	HSPN	2.71	55.0	20.3
2	IgA-N	2.55	185	72.5
3	NS	3.33	269	80.8
4	SLE-MN	9.23	417	45.2
5	NS	20.1	463	23.0
6	IgA-N	4.21	333	79.1
7	MPGN	7.4	28.0	3.8
8	CRF	6.0	111	18.5
9	CNS	4.95	278	56.2
10	MPGN	12.6	17.0	1.4
11	HSPN	6.14	17.0	2.8
12	MN	10.8	259	23.9
13	FGS	10.8	57.0	5.3
14	FGS	31.4	2498	79.5
15	IgA-N	1.97	91.0	46.2
16	CRF	2.37	4.40	1.8
17	FGS	0.97	43.8	45.1
18	F-B2MG	1.56	44.8	28.7
19	CTA-I	1.48	261	176
20	CNS	13.6	n.d.	n.d.
21	ALP	2.53	n.d.	n.d.
22	CNS	1.45	n.d.	n.d.
23	IgA-N	1.45	n.d.	n.d.
24	CRF	0.72	n.d.	n.d.
25	IgA-N	2.89	n.d.	n.d.
Median		3.11	49.9	19.4
Range		0.97-31.4	0-2498	0-176

* Biotinidase activity was determined by measuring the hydrolysis rate of the enzyme substrate (BAQ).

n.d.: Not detectable

**

HSPN: Henoch-Schönlein purpura nephropathy; *IgA-N:* IgA nephropathy *NS:* nephrotic syndrome (idiopathic); *SLE-MN:* membranous lupus nephritis *MPGN:* membranoproliferative glomerulonephritis; *CRF:* chronic renal failure; *CNS:* congenital nephrotic syndrome; *MN:* membranous nephropathy; *FGS:* focal glomerulosclerosis; *F-B2MG:* familiar β-2 microglobulinuria; *CTA-I:* congenital tubular acidosis, type I; *ALP:* Alport syndrome.

proteins. After centrifugation and deproteinization, a portion (10 µl) of the clear supernatant was injected into the HPLC system. The product of AQ was measured at an excitation wavelength of 350 nm and an emission wavelength of 550 nm. AQ was assayed as previously described [7] with the exception of solvent A where a 0.1 M-sodium phosphate buffer (pH 2.1) was used instead of a 0.1 % aqueous TFA solution.

To assay the effect of urine volume on AQ liberation rate, each volume of urine (5, 10, 15, 20, 25, 30 µl) was mixed with 70 µl of substrate solution and a volume of distilled water (25, 20, 15, 10, 5, 0 µl) in order to maintain a final reaction volume of 100 µl (final substrate concentration : 28 µM). The mixtures were incubated at 37 °C for 1.5 h. Methanol (200µl) was added to stop the reaction and the mixtures were processed as described above.

For the time-course study on the liberation of AQ, incubation times of 1, 2, 3, 4, 5, and 6 h were used on one representative urine specimen (patient No. 2) and compared to boiled urine samples (tubes were immersed in a boiling water bath for 3 minutes). All the assays were run in duplicate and each run was repeated three times.

Biotinidase activity was calculated as follows: [AA = amount of AQ in 10 µl] (pmol) = [peak height (mm) of AQ in sample/peak height (mm) of 100 pmol AQ] x 100 (pmol). Enzyme activity ($pmol \cdot min^{-1} \cdot ml^{-1}$) = AA (pmol)x [0.3 (ml)/0.01 (ml)]/reaction time (min)/0.01 (ml) of urine. Specific activity was expressed as pmol AQ liberated $\cdot min^{-1} \cdot mg^{-1}$ of protein. If interference was detected, peak height of the blank sample (i.e. incubated without BAQ) was substracted from peak height of the sample incubated in the presence of BAQ prior to the calculation of enzyme activity as described above.

Protein content: Protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

RESULTS

A typical chromatogram from the patient's urine is shown in Fig.1, A. Interference was usually not detected in the chromatograms (Fig.1, B). Hydrolytic reaction product (AQ) appeared as a sharp peak (mean retention time: 1.6 min). The amount of AQ increased linearly with the volume of urine (5-30 µl) and the amount of urine added to the reaction mixture (Fig. 2). The hydrolysis of BAQ, as a function of time, by one urine sample (patient no. 2, Table I) is shown in Fig. 3. The hydrolytic reaction proceeded linearly for a minimum of 6 h while boiled urine did not hydrolyze the substrate at all. Mean intra- and inter-assay coefficients of variation were 1.2 % (n = 6) and 2.6% (n = 6), respectively.

The results of the application of the assay method to the urine samples of patients with renal disease are shown in Table I. Since the data showed a skewed distribution, the results are presented as median and range values. The patient samples exhibited biotinidase activity with the exception of six of the patients (median: 49.9 pmol · min^{-1} · ml^{-1} of urine; range: 0 - 2498 pmol · min^{-1} · ml^{-1}). Median specific activity was 19.4 pmol · min^{-1} · mg^{-1} of protein (range: 0 - 176 pmol · min^{-1} · mg^{-1}). On the other hand, none of the 40 control subjects urine specimens exhibited detectable BAQ-hydrolyzing activity, although incubation times of the reaction mixture were maintained for up to 18 h (data not shown).

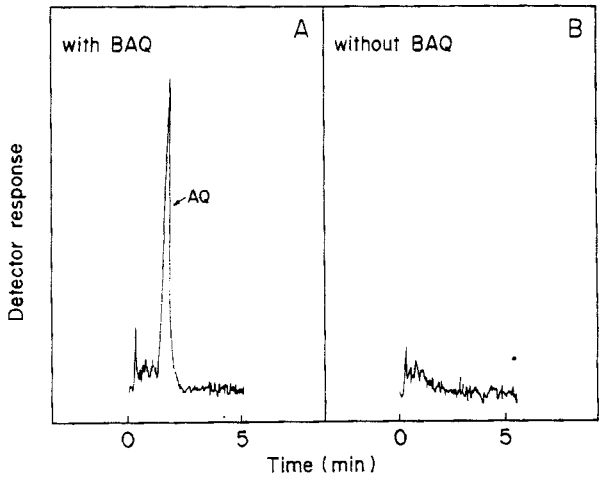

Fig. 1. Typical example of a chromatogram showing the hydrolysis of BAQ by one urine sample (patient no. 2, Table I). Panel A: urine, + BAQ; Panel B: urine, -BAQ (blank). Incubation time was 90 min. Conditions were as described in Materials and Methods.

DISCUSSION

A recently devised HPLC-fluorimetric assay, using BAQ as substrate, was applied to the determination of biotinidase activity in the urine. This assay method is relatively free from interference, due to prior separation by HPLC, and enables the detection of biotinidase activity in turbid specimens [6, 7]. The linear volume- and time- dependency of the hydrolytic reaction product (Figures 1 and 2) indicates that the urine from patients with proteinuria due to renal disease contained enzyme activity, i.e. BAQ hydrolase (biotinidase) activity.

In the patients with renal disease, median biotinidase activity was approximately 1/7 of the mean reported for human serum [5] and 2.6 times that reported for breast milk [6]. However, compared to the data

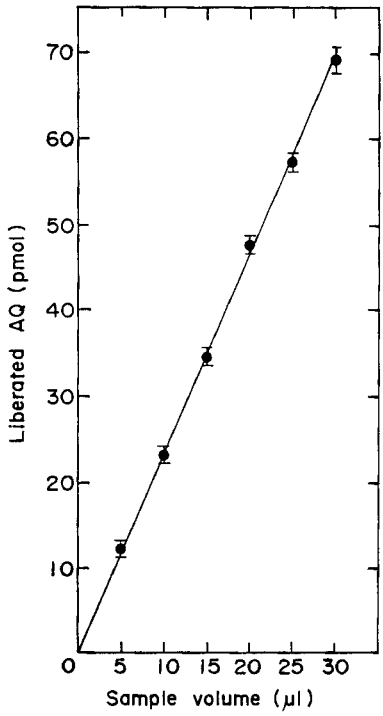

Fig. 2. Liberation of product (AQ), as a function of volume, for one urine sample (patient no. 2, Table I). Other conditions were as described in Materials and Methods. (Means ± SD; $n = 6$).

reported for serum and milk, enzyme activities in the urine were characterized by a wider dispersion. Moreover, despite the presence of proteinuria, biotinidase activity was not detected in six out of 25 (24 %) of the patient samples. Although the reason for the latter finding is not clear at this time, either considerably different etiologies of the renal disorders or varying degrees of glomerular/tubular damage may account for the reported skewness to the distribution. However, possible limits in the sensitivity of the assay or differences due to enzyme storage stability also need to be evaluated.

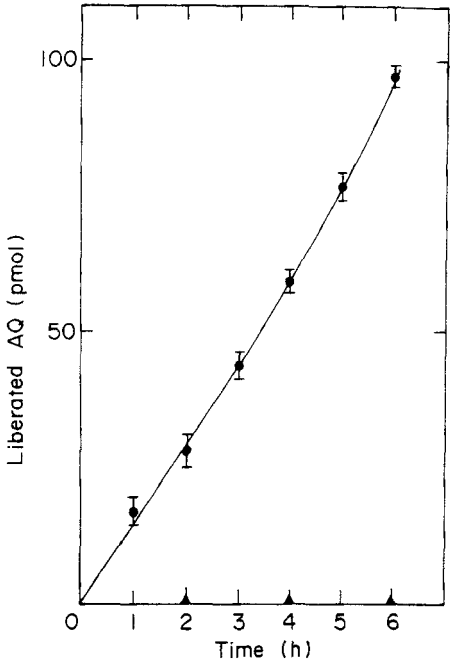

Fig. 3. Liberation of product (AQ), as a function of time, for one urine sample (patient no. 2, Table I). Liberated AQ per injection volume (10 µl) is indicated (circles: urine samples; triangles: boiled samples). Other conditions were as described in Materials and Methods . (Means ± SD; $n = 6$)

The actual origin of the biotinidase activity detectable in the urine of the majority (76%) of the urine samples from the renal patients remains to be established: although leakage of the serum enzyme protein through the glomerulus seems a plausible explanation, it remains to be tested.

Finally, our findings firstly indicates the urine from some patients with proteinuria as a novel potential source for purifying biotinidase.

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