Journal of Chromatography, 617 (1993) 29–35
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6876

Improved high-performance liquid chromatographic determination of biotinidase activity with biotinyl-6-aminoquinoline as substrate

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(First received October 16th, 1992; revised manuscript received April 8th, 1993)

ABSTRACT

An improved high-performance liquid chromatographic assay method for biotinidase activity was developed using the fluorimetric substrate biotinyl-6-aminoquinoline, which was found to be more specific than the biotinyl-4-aminobenzoate previously used. The new method measures the intensity of the fluorescent signal at wavelengths (excitation 350 nm; emission 550 nm) longer than those (excitation 276 nm; emission 340 nm) for 4-aminobenzoate. The analysis of fluorescence in the visible spectrum reduced considerably the number of interfering peaks compared with analysis in the ultraviolet region. This method also made it possible to measure the biotinidase activity directly in samples usually difficult to calculate, such as human and bovine milk or porcine serum; the use of biotinyl-6-aminoquinoline allowed the analysis of the enzyme reaction in milk and porcine serum without pretreatment or dialysis. Stoichiometric increase and decrease of the substrate and product, respectively, were demonstrated. Michaelis constants for biotinyl-6-aminoquinoline were measured at various stages of partial purification. Because the solubility of these synthetic substrates in the aqueous phosphate buffer is limited, the determination of both Michaelis constant and maximum velocity by extrapolation may be helpful for the characterization of the kinetics of biotinidase.

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0378-4347/93/$06.00

INTRODUCTION

Biotinidase (EC 3.5.1.12) is a hydrolytic enzyme, which cleaves biocytin to produce biotin and lysine. The enzyme also cleaves some artificially synthesized biotin derivatives, such as biotin 4-amidobenzoate (BPAB) [1] and biotin 6-amidoquinoline (BAQ) [2].

A sensitive fluorimetric high-performance liquid chromatographic (HPLC) biotinidase assay method, which uses BPAB as substrate, was previously developed by us [3]. The method is relatively free from interference due to prior separation by HPLC, and enables the detection of biotinidase activity in turbid specimens, such as human breast milk [4]. The method measures the intrinsic fluorescent activity of one of the PAB products at a relatively short UV wavelength (excitation 276 nm; emission 340 nm).

Another fluorescent synthetic substrate using BAQ for the biotinidase assay was previously described by Wastell *et al.* [2]. In their method the intensity of the fluorescent signal is measured at a pair of wavelengths (excitation 350 nm; emission 550 nm) longer than those of 4-aminobenzoate (PAB) (excitation 276 nm; emission 340 nm).

In the present study, this fluorescent biotinidase substrate of BAQ was combined with our earlier reported HPLC–fluorimetric biotinidase assay method [3,4]. The changes in the detection wavelength and in the gradient programme for HPLC analysis were sufficient for the application. The analysis time for each sample was as short as 10 min. This method, using BAQ instead of BPAB, resulted in a remarkable improvement in the accuracy, because detection can be performed in the visible region. This analytical method proved to be an accurate way to determine biotinidase activity even at very low levels. Some results of kinetic studies are also presented.

EXPERIMENTAL

Chemicals and reagents

BAQ and BPAB sodium salt were purchased from Sigma (St. Louis, MO, USA). 6-Aminoquinoline (AQ) hydrochloride was obtained from Tokyo Kasei (Tokyo, Japan). AQ was obtained from Aldrich (Milwaukee, USA). Trifluoroacetic acid (TFA, amino acid sequencing grade), methanol (liquid chromatography grade) and other chemicals were highly pure substances provided by Wako (Osaka, Japan).

Specimens

Human serum was obtained from the National Children's Hospital (Tokyo, Japan). Human milk was donated by the Tokyo Boshi-hoken-in Hospital (Tokyo, Japan). Bovine milk was a gift from Tokyo University of Agriculture (Tokyo, Japan) and Nihon University (Tokyo, Japan). Porcine serum was purchased from Pel-Freez Biologicals (Rogers, AR, USA). The specimens were stored at −80°C.

Instruments

A Waters Model 600 HPLC system (Waters Assoc., Milford, MA, USA) with a gradient elution unit was used. The column was a 50 mm × 4.6 mm I.D. stainless-steel tube packed manually with spherical 5-μm silica gel particles, chemically bonded with octadecylsilane (ODS) (Develosil ODS, Nomura Chemical, Aichi, Japan). The sample injection unit used was a diaphragm-type injector (Model U6K, Waters) with a 2-ml loop. Detection was carried out with a fluorimeter (F-3000, Hitachi, Tokyo, Japan) using a flow-through cell (cell volume 18 μl). Fluorescence spectra were obtained by this fluorimeter using an usual rectangular quartz cell.

Biotinidase assay

The enzyme assay procedure was essentially as described previously [3,4]. BPAB was dissolved at 0.275 m*M* (106 mg/l) in 0.1 *M* sodium phosphate buffer (pH 7.0) containing 1 m*M* (452 mg/l) Na_4EDTA and 10 m*M* (781 mg/l) 2-mercaptoethanol. BAQ was dissolved at 0.044 m*M* (16.3 mg/l) in the above phosphate buffer. Substrate containing 0.045 ml of the reaction buffer was mixed with 0.005 ml of enzyme solution. Thus, the reaction mixture (0.05 ml) contained 0.000734 mg (1.98 μmol) of BAQ, 0.020 mg of EDTA and 0.035 mg of 2-mercaptoethanol. The

reaction proceeded for an appropriate time at 37°C, and was stopped by adding 0.10 ml of methanol: the reaction mixture was diluted threefold with methanol in order to precipitate the enzyme proteins. After centrifugation and deproteinization, a portion (0.01 ml) of the clear supernatant (0.15 ml) 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 [2,5].

Biotinidase purification

Biotinidase in serum and milk was partially purified either by dialysis against 0.1 *M* sodium phosphate buffer (pH 7.0) overnight at 4°C, or by ammonium sulphate precipitation with 35–55% saturation. Further purification using DEAE, Sephadex G-200 and HPLC hydroxyapatite chromatography were also performed for human and bovine milk, as well as porcine serum.

Kinetic study

Michaelis constants (K_m) were determined by the calculation method of Lineweaver and Burk [6]. The substrate concentrations were 40, 20, 15, and 10 μM.

Protein content

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

RESULTS AND DISCUSSION

The HPLC biotinidase assay has been used in our laboratory since 1986 [3]. The method prevents interference due to turbidity, and has been successfully applied to human milk [4] and various tissues, such as homogenates of guinea-pig livers [7]. However, because the wavelengths that we used for the detection of PAB were similar to those of various nucleotides, the possibility of such interference remained. Indeed, the retention times of nucleotides in reversed-phase HPLC separation are very close to those of PAB. Therefore, we used a more suitable fluorimetric substrate, BAQ [2], for the biotinidase HPLC assay.

As shown in Fig. 1, AQ shows maximum excitation at 350 nm and maximum emission at 550 nm in acidic aqueous 0.1% TFA solution (solvent A of refs. 1 and 2; pH 2.0). The wavelengths of maximum intensities in acid solution appears to be the same as those observed in neutral solution [2,5].

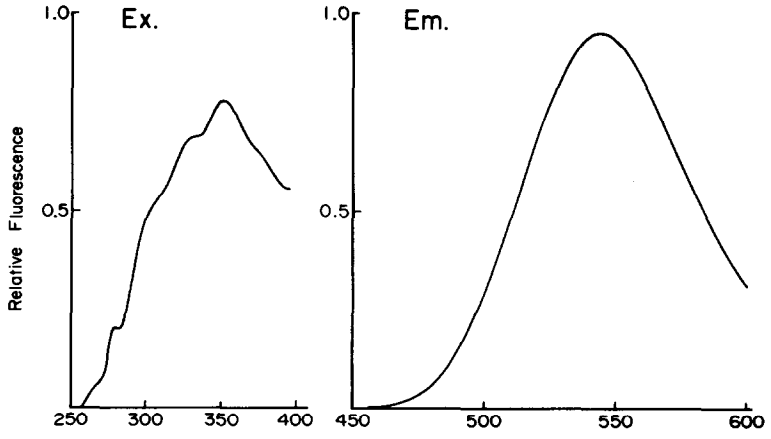

Fig. 1. Fluorescence spectra of 6-aminoquinoline (AQ) in aqeuous 0.1% trifluoroacetic acid solution. The concentration of AQ was 0.025 m*M*. Excitation spectra were obtained at 550 nm, and emission spectra at 350 nm. Other conditions were as described in Experimental.

TABLE I

ELUTION PROGRAMMES ROUTINELY USED FOR BAQ AND BPAB HYDROLASE ACTIVITIES

Proportioning valves were used; solvent A, 0.1% aqueous TFA solution; solvent B, methanol.

Time (min)	Flow-rate (ml/min)	Solvent A (%)	Solvent B (%)
Programme 1: for BAQ hydrolase activity (AQ assay)			
0.0	3.00	100	0
1.5	3.00	0	100
3.50	3.00	0	100
5.01	3.00	100	0
Programme 2: for BPAB hydrolase activity (PAB assay)			
0.0	1.00	100	0
3.0	1.00	100	0
3.01	2.00	0	100
5.00	3.00	0	100
5.01	3.00	100	0
8.00	3.00	100	0
8.01	1.00	100	0

Wastell *et al.* [2] measured the biotinidase activity at pH 6.5. However, marked changes in the fluorescent intensity were observed between pH 6 and 7, which might induce a slightly larger coefficient of variation (C.V.), 2.6%, than that (1.1%) of Knappe *et al.* [1]. On the other hand, we measured the fluorescence of AQ in acidic aqueous TFA eluent, and found that the activity was stable.

AQ was eluted at a retention time of 2.65 min in the HPLC system using the gradient programme 1 (Table I). Programme 2 for the PAB determination is also shown for reference (Table I). Therefore, the same HPLC system can be used for both PAB and AQ assays by changing only the elution programme and the detection wavelengths. The detection limit for AQ was 2 pmol at a signal-to-noise ratio (S/N) of 3 in this system using fluorimetric detection at an excitation wavelength of 350 nm and an emission wavelength of 550 nm. The S/N for AQ was 6 with 4 pmol of the compound, compared with an S/N of 3 with PAB under the same analytical conditions. The detection threshold could thus be halved. A linear relationship between the injected amount of AQ and the peak height was observed at the concentration range 2–128 pmol (2, 4, 8, 16, 32, 64, 128 pmol). One cycle of analysis time corresponded to 10 min. The intra-assay C.V. was 0.8% ($n = 6$) when 50 pmol of AQ were used.

The biotinidase reaction was studied using BAQ as the substrate. The results of a time-course study using human serum and various substrate

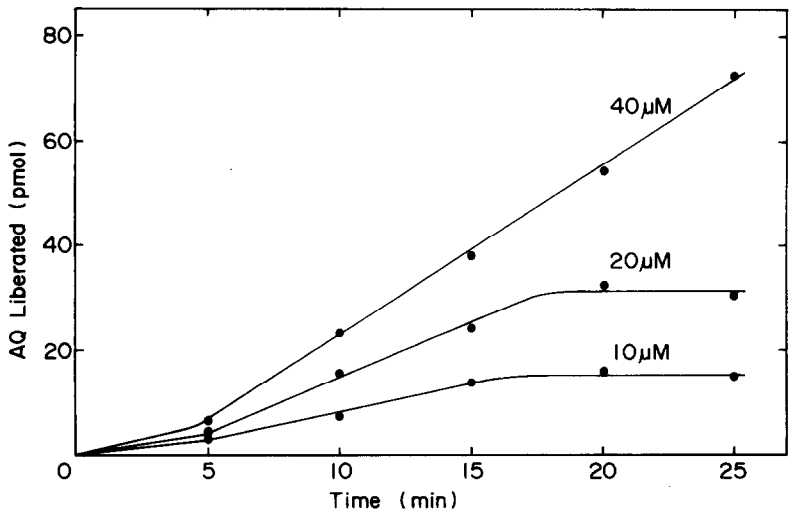

Fig. 2. Time-course study of BAQ hydrolysis using human serum. The reaction mixture (0.5 ml) was incubated at 37°C. After each time interval a 0.05-ml aliquot was withdrawn and assayed for liberated AQ as described in Experimental.

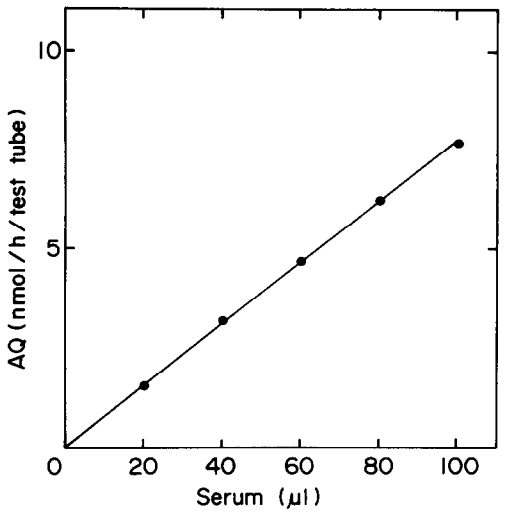

Fig. 3. Dependence of the biotinidase reaction on the volume of human serum. Different volumes (0.02–0.1 ml) of human serum were incubated in the reaction mixture (0.5 ml) for 60 min at 37°C. Liberated AQ was measured as described in Experimental.

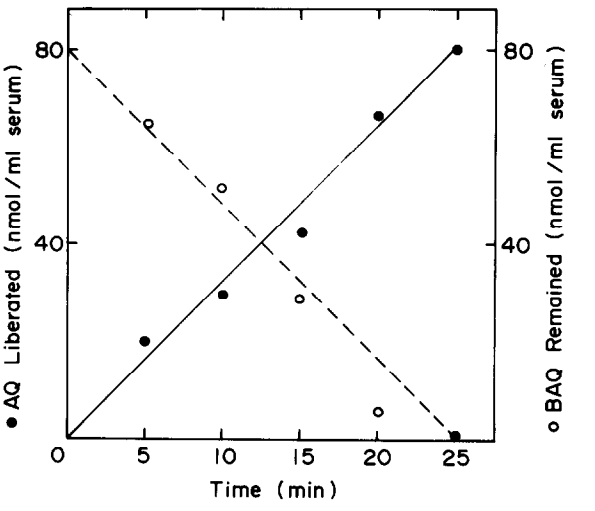

Fig. 4. Stoichiometry of the biotinidase reaction. The reaction proceeded as described in Fig. 2. In this case, product AQ and substrate BAQ were simultaneously analysed using a linear gradient (15 min) from solvent A to solvent B.

concentrations are shown in Fig. 2. Although a time lag was observed within 5 min, the reaction rate can be calculated with appropriate incubation times. Liberation of product AQ was linearly increased by incubation with an increasing volume of human serum (Fig. 3). The biotinidase reaction was found to proceed stoichiometrically (Fig. 4).

The new method was applied to the analysis of various biological fluids (human and porcine serum, human and bovine milk). Typical chromatograms for porcine serum are shown in Fig. 5, with reference to the substrate BPAB. Porcine serum exhibited AQ production activity after 10 min of incubation at 37°C (Fig. 5A). No interfering peaks from porcine serum were observed in the AQ analysis, whereas at least three interfering peaks were noticed when the substrate BPAB was used (Fig. 5B). The effect of the dialysis is shown in Fig. 5C and D. The interfering peaks previously recorded with the substrate BPAB disappeared after dialysis (Fig. 5D). Therefore, we were able to analyse porcine serum samples without pretreatment by substrate BAQ. On the other hand, no interference was observed in human serum (data not shown). The reason for the difference in dialysable interfering peaks between porcine and human samples remains to be elucidated. However, dietary metabolic differences due to nucleotides may be involved.

The number of interfering peaks was remarkably reduced with milk specimens when substrate BAQ was used (data not shown). A typical example of the use of BAQ for the determination of the biotinidase activity in milk specimens of human and bovine is shown in Fig. 6.

The results of mechanistic studies using BAQ as substrate with several different partially purified biotinidases are summarized in Table II. Although enzyme purification was not perfect, these data suggest that there are genetic and individually related differences in the mechanistic parameters of the biotinidase activity. In terms of species differences, human and porcine serum showed similar kinetics. On the other hand, a ten-fold larger K_m was detected in human milk compared with bovine milk. This difference was not observed in the reaction mixture without 2-mercaptoethanol: 16 and 31 μM for human and bovine milk biotinidase, respectively. Although activity was

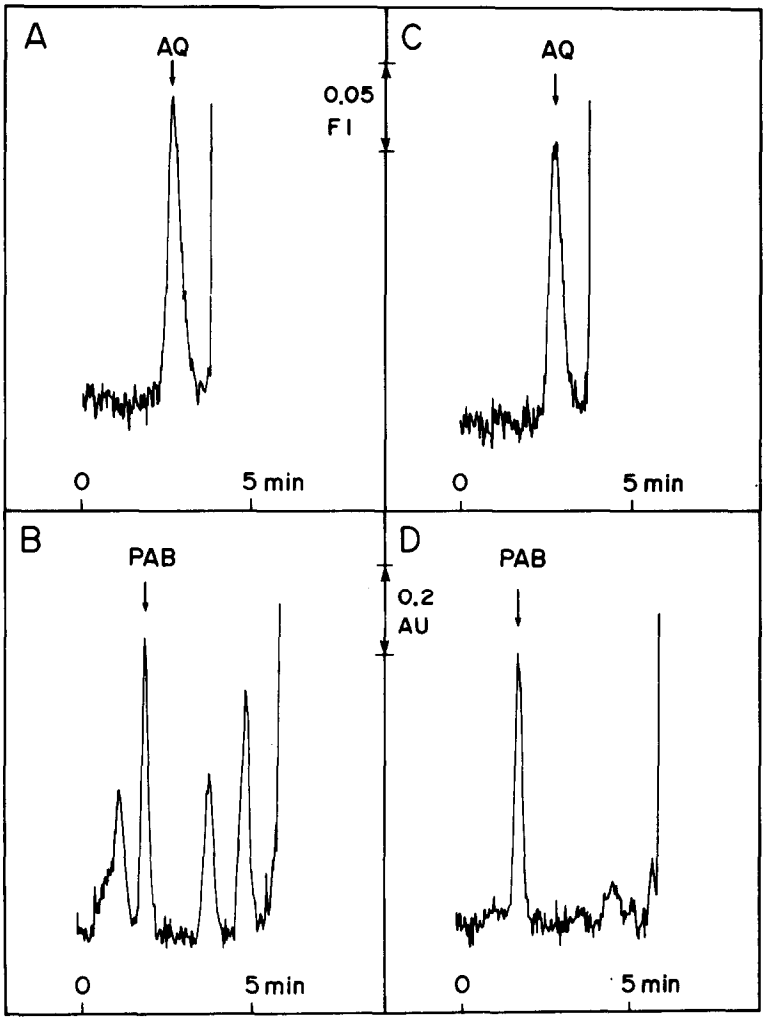

Fig. 5. Typical example of determination for applications with porcine serum biotinidase assay using BAQ as substrate in comparison with assay using BPAB as substrate. Porcine serum, human serum, dialysed porcine serum and dialysed human serum were used. Dialyses were performed overnight against 0.1 *M* sodium phosphate buffer (pH 7.0) at 4°C. Other conditions for analysis were as described in Experimental. (A) Human serum assayed with BAQ as substrate. (B) Porcine serum assayed with BPAB as substrate. (C) Dialysed human serum assayed with BAQ as substrate. (D) Dialysed porcine serum assayed with BPAB as substrate.

strongly reduced, human milk biotinidase showed a K_m value of 14.9 μM in the reaction mixture containing 2-mercaptoethanol after acetone treatment and ammonium sulphate purification.

Thus, we have demonstrated that BAQ can be applied in the HPLC biotinidase assay of turbid specimens, such as human and porcine milk and porcine serum, without pretreatment. BAQ is also suitable for estimating Michaelis constants.

ACKNOWLEDGEMENTS

This work was supported by a grant-in-aid from the Ministry of Health and Welfare of Japan, and performed through Special Coordination Funds of the Science and Technology Agency of the Japanese Government. We are grateful to Dr. Masahiko Hirano, Tokyo University of Agriculture, and to Takayuki Watanabe MVS, National Children's Medical Research Center, for their generous assistance.

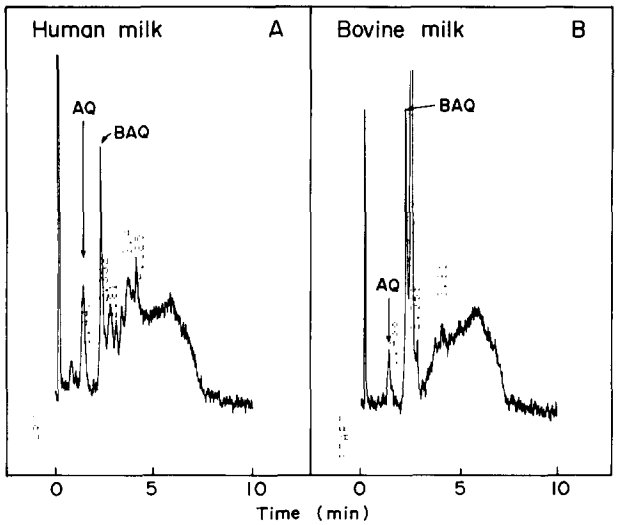

Fig. 6. Typical example of biotinidase assay using BAQ as substrate when applied to human milk (A) and bovine milk (B). Milk (0.01 ml) was incubated with BAQ containing substrate buffer (BAQ concentration at 0.04 mM; 0.09 ml) for 18 h at 37°C. Other conditions were as described in Experimental.

TABLE II

DETERMINATION OF MICHAELIS CONSTANTS FOR BAQ

Specimen[a]	K_m (μM)	V_{max} (pmol/min/mg of protein)
Human serum	52	263
	17	130
	16	98
Human serum biotinidase	12	2468
Porcine serum	15	526
Human milk	256 (16)[b]	213 (18.7)[b]
Bovine milk	41 (31)[b]	53 (12.6)[b]

[a] Human serum was purified by ammonium sulphate precipitation (35–55%), and human serum biotinidase by ammonium sulphate precipitation, DEAE-Cellulofine, Sephadex G-200, hydroxyapatite HPLC, and high-performance gel-permeation chromatography [8]. Porcine serum, human and bovine milk were purified to the hydroxyapatite step.

[b] Values in parentheses are Michaelis constants measured without 2-mercaptoethanol.

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