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Note

Determination of biotinidase activity by liquid chromatography with fluorimetric detection

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Biotinidase (EC 3.5.1.12) is a hydrolytic enzyme, which cleaves biocytin to produce biotin and lysine. The enzyme also cleaves some artifically synthesized biotin derivatives, e.g. N-biotinyl-*p*-aminobenzoic acid (biot-PAB) [1, 2].

Biotinidase activity has been determined by measuring biotin liberated from biocytin using the microbiological method [3]. However, this biological method requires a longer time and a more tedious procedure than other chemical methods.

The colorimetric method of Knappe et al. [2], which measures the derivative of the *p*-aminobenzoic acid (PAB) liberated from biot-PAB, has been routinely employed for biotinidase assay. In the method of Knappe et al., the liberated PAB is further diazotized to yield the pink chromophore (azo dye) described by Bratton and Marshall [4]. However, chloride ions and other chemicals discussed by Bratton and Marshall interfere with the formation of this azo dye.

The fluorimetric method of Wastell et al. [5], which uses a fluorescent derivative of biotin (biotinyl-6-aminoquinoline), has been published recently. The method seems to give relatively higher values for the coefficient of variation (C.V. = 2.6%) than the method of Knappe et al. [5] (C.V. = 1.1%). In addition, it requires the synthesis of biotinyl-6-aminoquinoline.

We describe here a simple and stoichiometric determination of biotinidase activity by simultaneously measuring the liberated PAB and residual biot-PAB by high-performance liquid chromatography (HPLC) with a fluorimeter.

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EXPERIMENTAL

Chemicals and reagents

D-Biotin, methanol (HPLC grade), trifluoroacetic acid (amino acid sequencing grade, TFA) were obtained from Wako (Osaka, Japan) and PAB was from Kanto (Tokyo, Japan).

The substrate (biot-PAB) was prepared from biotin and PAB according to the method of Wolf et al. [6]. The purity of the synthesized product was assessed using the HPLC analysis as described by Chastain et al. [7], and our method described below. Biot-PAB was dissolved in 0.1 *M* potassium phosphate buffer (pH 6.0) and used as a substrate solution.

High-performance liquid chromatography

The liquid chromatograph was a Hitachi 655A-11 (Hitachi, Tokyo, Japan) equipped with a gradient elution unit (655A-71: proportioning valve mixing method), a sample injector (Rheodyne Model 7125 with a 100-μl loop), and a fluorescence spectrophotometer (F-1000). The gradient elution programme and the electrical signal from the detector were controlled and assessed by a data processor Model 655-61 (Hitachi).

The column used as Nucleosil $5C_{18}$ (250 × 4.6 mm I.D., Macherey, Nagel, Düren, F.R.G.) with a guard column (Develosil ODS, Nomura, Aichi, Japan; 10 × 4.0 mm I.D.).

Simultaneous determination of PAB and biot-PAB was carried out using a linear gradient from 100% of solvent A (aqueous 0.1% TFA) to 100% of solvent B (methanol). We also included a flow-rate programme to reduce the column-inlet pressure: Table I shows a typical programme for which the total analysis time was ca. 45 min.

We devised a faster analytical method for PAB determination, using an ODS column (50 × 4.0 mm I.D., Develosil ODS) and an isocratic elution with solvent A (aqueous 0.1% TFA) at a flow-rate of 2.0 ml/min. Using this system, the analysis time for PAB in purified samples was reduced to 5 min.

TABLE I

TYPICAL ELUTION PROGRAMME ROUTINELY USED IN THIS STUDY

Proportioning valves A and B were used. The column back-pressure ranged from 100 to 250 kg/cm^2.

Time (min)	Flow-rate (ml/min)	Solvent A (%)	Solvent B (%)
0.0	1.00	100	0
0.1		98	2
3.0		95	5
6.0		70	30
10.0	0.80	50	50
25.0		35	65
25.1	1.00	0	100
26.0		0	100
26.1		100	0

Detection of PAB and biot-PAB was carried out by measuring their intrinsic fluorescent activity. An excitation wavelength of 276 nm and an emission wavelength of 340 nm were used.

Enzyme assay

The enzyme-containing solution (0.5 ml) was mixed with an equal volume of the substrate solution (usually 0.15 m*M* biot-PAB) in 0.1 *M* potassium phosphate buffer (pH 6.0). The reaction mixture was incubated at 37°C. At adequate time intervals, a portion of the reaction mixture was withdrawn and heated to boiling in order to stop the reaction. In the case of serum samples, the boiled reaction mixture was cooled to room temperature, and two volumes (2 ml) of methanol were added. The mixture was stirred and mixed. After centrifuging at 1500 *g* for 5 min at 4°C, the clear supernatant was withdrawn and filtered through a disposable membrane filter (Ekicrodisc 3, 0.45 μm pore size, Gelman Sciences, MI, U.S.A.). A portion of this filtrate (usually 2—10 μl) was injected into the HPLC system. In the case of an enzyme solution partially purified by DEAE-cellulose, this deproteinization procedure by methanol was omitted. This assay (reaction volume 2.0 ml) permits the use of injection volumes as low as 20 μl (10 μl of substrate solution and 10 μl of serum).

Biotinidase was also assayed colorimetrically according to the method of Knappe et al. [2]. Protein was determined by the method of Lowry et al. [8].

RESULTS AND DISCUSSION

According to the method of Knappe et al. [2], the PAB formed was further processed to obtain the azo dye. We have attempted to measure directly the liberated PAB and residual biot-PAB using HPLC.

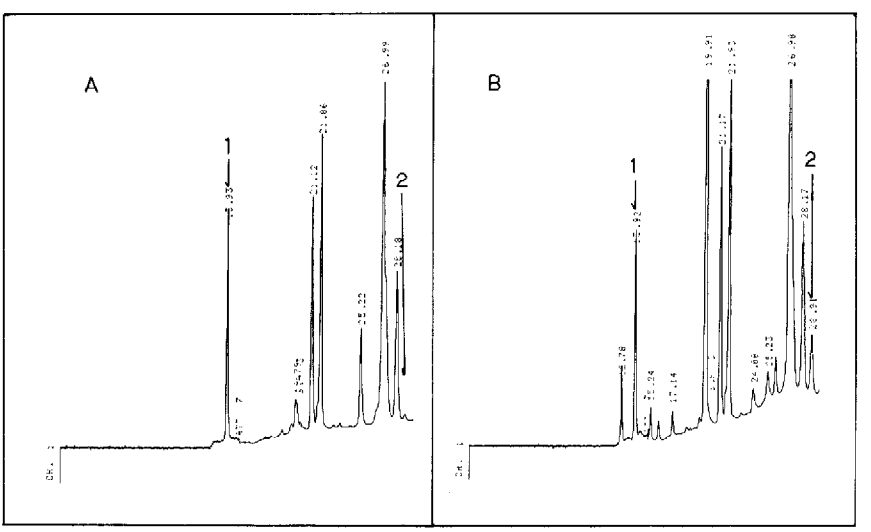

Fig. 1. Chromatograms of samples. (A) Partially purified enzyme solution from serum by a DEAE-cellulose column; (B) serum sample. Peaks: 1 = PAB; 2 = biot-PAB. For analytical conditions see Experimental.

By using a Nucleosil $5C_{18}$ column and the gradient programme shown in Table I, the two standards of PAB and biot-PAB were eluted at 14 min and 29 min, respectively. The relationship between the amount of PAB and biot-PAB injected and the peak areas was linear in the concentration range 11—132 pmol (11, 22, 44, 88 and 132 pmol).

We applied this method to serum and a partially purified enzyme solution. Typical examples of the chromatographic separations are shown in Fig. 1.

The C.V. of the PAB determination was below 2.0%: serum A, 1.4%; serum B, 2.0%; purified enzyme solution, 1.0% (n = 3). As expected, purified sample gave lower C.V. values than sera. The C.V. for the biot-PAB determination was also below 2.0%: serum A, 1.1%; serum C, 0.75% (n = 3).

The recovery at a concentration of 2.69 μg PAB from 1 ml of serum was 87.3% (n = 3, C.V. = 1.3%). The recovery of biot-PAB (125 μg) from 1 ml of serum was 99.1% (n = 3, C.V. = 8.5%). These results indicate that PAB and

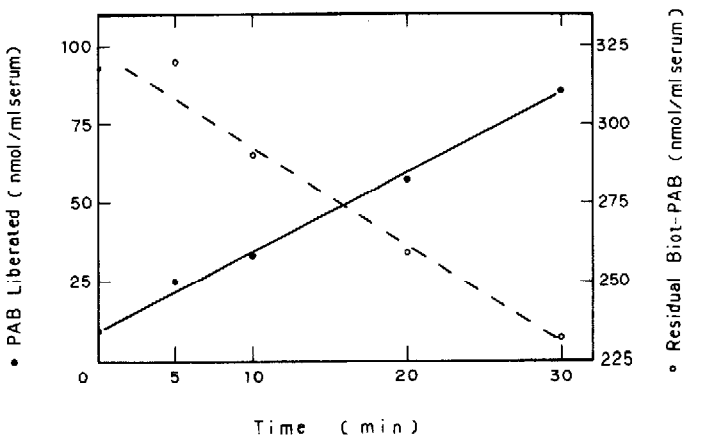

Fig. 2. Hydrolysis of biot-PAB in a serum sample as a function of time. Conditions as in Experimental; substrate concentration, 106 μ*M*.

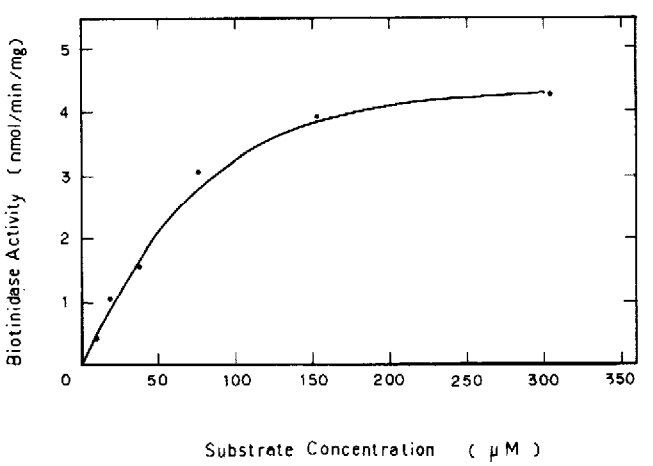

Fig. 3. Effect of the concentration of biot-PAB (substrate) on the rate of hydrolysis. Assay conditions as in Experimental. Biotinidase activity was measured after a 5-min incubation. Partially purified enzyme with 0.06 mg of protein per ml was used.

biot-PAB can be determined quantitatievly in serum samples using this method. Other procedures for deproteinization, using acetonitrile, trichloroacetic acid (TCA), perchloric acid (PCA), of 5-sulphosalicylic acid treatment, were found to give low yields.

This method was applied to the determination of the activity of biotinidase in human serum. The time-course of hydrolysis of the substrate (biot-PAB) is as shown in Fig. 2. Interferences from sulphonamide and other chemicals were observed by other researchers [9]. However, these compounds did not interfere in our chromatographic method.

The effect of the substrate concentration on the rate of the enzyme reaction was studied and the results are shown in Fig. 3. The Michaelis constant (K_M) for biot-PAB was found to be 50 μM, which is similar to the value determined by Chauhan et al. [10]. However, Craft et al. [11] and Wolf et al. [12] reported the K_M value to be 10 μM, determined by the colorimetric method of Knappe et al. [2]. This discrepancy might result from the assay end-point at 30 min. The rate of production of PAB is already at the plateau region at 30 min (data not shown), so the enzyme substrate (biot-PAB) is not available after 30-min incubation. Our method can be applied to kinetic and stoichiometric studies, and micro enzyme assay.

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