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Note

Determination of free biotin in plasma by liquid chromatography with fluorimetric detection

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Biotin (vitamin H) is a known co-enzyme for carboxyl translocation reactions in enzymes such as pyruvate carboxylase, acetyl-CoA carboxylase, propionyl-CoA carboxylase and β-methylcrotonyl-CoA carboxylase [1]. Biotin may exist either in the free form or covalently bound to apoenzyme of biotin-dependent enzymes. It has been suggested that the plasma-free biotin level is regulated by intestinal simple diffusion [2].

Biotin has long been determined by a microbiological method [3], which is tedious and time-consuming. Another chemical method, based on isotope dilution [4], uses avidin and radioisotopic biotin. However, isotope handling requires a restricted area and this method does not discriminate biotin from its analogues.

Recently, the derivatization of biotin with the fluorescent reagent 9-anthryldiazomethane (ADAM) has been described [5]. Using this reagent, we have developed a new analytical method for assaying free biotin in human plasma.

EXPERIMENTAL

Chemicals and reagents

Methanol and acetonitrile were HPLC grade from Wako (Osaka, Japan). Trifluoroacetic acid (TFA) was amino acid sequencing grade from Wako. Ethyl acetate, ethanol and aqueous ammonia (specific gravity 0.90) were reagent grade from Kanto (Tokyo, Japan). 9-Anthryldiazomethane (ADAM) was from Funakoshi Pharmaceutical (Tokyo, Japan) [6,7] and stored at $-20°C$. Before use, it was dissolved in ethyl acetate at a concentration of 0.1% (w/v). The reference biotin was purchased from Nakarai (Kyoto, Japan), and dissolved in methanol at a concentration of 0.1 mg/ml.

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High-performance liquid chromatography

The chromatographic apparatus (Hitachi, Tokyo, Japan) consisted of a 655A-11 liquid chromatograph equipped with a gradient elution accessory (655-71), a sample injector (Rheodyne Model 7125) with 100-μl loop and a fluorescence spectrophotometer (F-1000, Hitachi). The step-wise gradient programme and the detector signal were processed by a Model 655-61 data processor (Hitachi).

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

Biotin-ADAM ester was analysed isocratically using a mixture of 52% solvent A (0.8% TFA in water) and 48% solvent B (0.1% TFA in acetonitrile) for 30 min and then 100% solvent B for 3 min. The 3-min elution with 100% solvent B served to eliminate the late-eluting compounds and to ensure reproducibility. The total HPLC analysis time was 45 min. The flow-rate was 1.0 ml/min. After analysis of ten samples, the column was washed with chloroform–methanol–ethyl acetate (1:1:1, v/v/v). Biotin-ADAM was detected fluorimetrically using an excitation wavelength of 365 nm and an emission wavelength of 412 nm.

Preparation of biotin ester of ADAM from plasma

A 1-ml sample of plasma diluted with 1 ml of distilled water, then ultrafiltrated through an ultrafiltration membrane (DIAFLO PM 10, Lot AE 02105B, Amicon, Danvers, MA, U.S.A.) under nitrogen at a pressure of 2 kg/cm^2. This filtration procedure took ca. 30 min. Then 1 ml of the filtrate was mixed with 3 mg of activated charcoal (Darco G type, Wako). The mixture was centrifuged in an Eppendorf microcentrifuge at 10 000 *g* for 5 min. The precipitate was extracted with 1 ml of 5% ammonia solution (specific gravity 0.90) in ethanol. The supernatant was separated from the activated charcoal by centrifuging at 10 000 *g* for 5 min. Then the solution was evaporated to dryness in a centrifugal evaporator at 45 °C for 20 min (Yamato, Tokyo, Japan). The dried sample was dissolved in 100 μl of methanol. This sample solution was added to 80 μl of 0.1% (w/v) ADAM solution in ethyl acetate. The mixture was allowed to stand for 1 h at room temperature, wrapped in aluminium foil. A 2-μl sample of this reaction mixture was injected into the HPLC system.

RESULTS AND DISCUSSION

Liquid chromatography

The prepared biotin ester of ADAM was separated on a reversed-phase column (Nucleosil $5C_{18}$) and detected as described in Experimental. A typical chromatogram of a plasma sample is shown in Fig. 1. A number of peaks that were observed in plasma specimens appeared to be mostly of plasma fatty acids. However, the peak corresponding to biotin was completely separated from other endogenous compounds. There was a linear correlation between the concentration of the ADAM derivative of biotin and the peak area in the concentration range 0.1–10 ng (correlation coefficient 0.9998, data not shown). This method was sensitive enough to measure the plasma level of biotin. Biotin was added to the plasma and

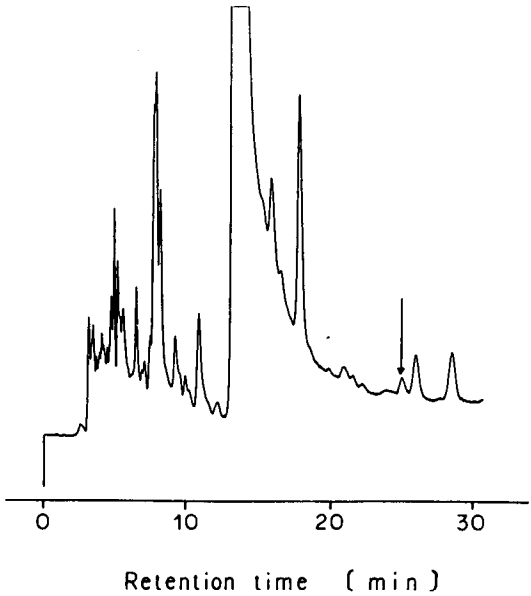

Fig. 1. Typical chromatogram of a plasma sample. The arrow indicates the peak of the biotin-ADAM ester. Analytical procedures were as described in Experimental.

was extracted as described in Experimental. The recovery data are shown in Table 1. Although the recovery was found to be low, the values were constant and reproducible. Therefore, this method was considered to be applicable to the measurement of plasma free biotin. The coefficient of variation of peak areas, determined from triplicate injection of 2-μl samples (25.2 ng/ml), was 12.3%.

TABLE I

RECOVERY OF BIOTIN

Sample volume, 1 ml of plasma. Extraction and analytical procedures as described in Experimental.

Amount added (μg)	Amount found (μg)	Recovery (%)
100	41.6	41.6
	36	36
	50.9	50.9
Mean ± S.D. (unbiased form)	42.8 ± 7.5	42.8 ± 7.5
Coefficient of variation (%)	17.6	17.6
1.0	0.36	36
	0.486	48.6
	0.63	63
Mean ± S.D. (unbiased form)	0.492 ± 0.135	49.2 ± 13.5
Coefficient of variation (%)	27.5	27.5

TABLE II

CONCENTRATION OF FREE BIOTIN IN PLASMA

Plasma No.★	Concentration★★ (ng/ml)
1	64.8
2	32.4
3	18.0
4	16.2
5	5.4
6	11.2
7	27.0
8	5.4
9	14.4
10	32.4
11	32.4
12	25.2
Mean ± S.D. (unbiased form)	23.7 ± 16.3

★Nos. 1–6 from the hospital; Nos. 7–12 from a blood supplying center.
★★Calculated by correcting for the recovery of 50%.

Measurements of plasma samples

Twelve plasma samples from healthy subjects were obtained from the hospital and a blood supplying center. As shown in Table II, free biotin levels ranged from 5.4 to 64.8 ng/ml (average 23.7 ng/ml). These values were relatively higher than the values previously reported (3.27 ng/ml by a chemical method) [8]. The described HPLC method is relatively simple, rapid and economical compared with other methods. Thus, it can be a useful tool in the clinical as well as the research laboratories.

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