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Determination of biotin (vitamin H) by the high-performance affinity chromatography with a trypsin-treated avidin-bound column

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In memory of the kind encouragement of my beloved daughter, Reiko Hayakawa (21 November 1979 – 1 February 2007).

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ABSTRACT

A method for measuring biotin by affinity-chromatography was developed using a trypsin-treated avidin silica gel column. Elution was by a linear gradient of propan-2-ol in an acidic phosphate buffer system containing 0.7 M NaCl (pH 2.4). Biotin was derivatized with 9-anthryldiazomethane (ADAM) to the fluorescent biotin-ADAM ester and a linear calibration line was obtained from 0 to 1.39 pmol with a detection limit of 69.5 fmol. Biotin was measured after hydrolysis in 2.25 M sulphuric acid for 1 h at 120 °C and the recovery for biocytin was 65.7 ± 2.53%, and hence a correction factor of 1.52 was used for the total biotin analysis. The recovery of added biotin from the serum was more than 98% using this correction factor of 1.52. Biotin was also measured in nutritional supplemental foods and foodstuffs, and we found that chicken egg yolk, "natto", rice bran, royal jelly, and dried yeast contained highest levels of biotin. Biotin was also found in ferments by *Bacillus natto*, yeast, and some acetic acid bacterium. Storage foods such as beans, nuts and eggs also contained abundant biotin. Biotin was also determined and replacement monitored in the serum of suspected biotinidase deficiency patients. This affinity-chromatographic method for biotin determination was shown to be a robust and reliable and is well suited for biochemical and nutritional research.

1. Introduction

High-performance liquid chromatographic (HPLC) determination method of biotin is important, since a linear calibration line through the origin is usually obtainable in this method. Previously, we proposed a method for determining plasma free-biotin by RP-HPLC with fluorimetric detection [1]. Biotin was derivatized by 9-anthryldiazomethane (ADAM) to the ester of fluorimetric biotin-ADAM, and detected fluorimetrically at an excitation wavelength of 365 nm and emission wavelength of 412 nm [1].

Recently, we found that an avidin-bound silica gel column was useful to measure the D-aspartic acid [2]. Because we successfully separated the derivatives of D- and L-aspartic acid using an avidin-bound HPLC column [2], we thought avidin, a biotin-binding protein, might also be applicable to assay biotin. We attempted to apply the avidin-bound gel to the biotin analysis, but separation of biotin peak from other interfering peaks of sample in the stable acidic alcoholic phosphate buffer system [3] was not apparent whereas trypsin treatment of the avidin-silica gel allowed their resolution. Furthermore, a column length of 3.3 cm was found to be suitable for demonstrating the symmetrical biotin-ADAM peak at a relatively low temperature.

In this text, applications of the trypsin-treated avidin-bound affinity column to the measurement of biotin in various foods,

Abbreviations: Bct, biocytin (ε-*N*-biotinyl-L-lysine); ADAM, 9-anthryldiazomethane; PBS, phosphate-buffered saline; RP-HPLC, reversed-phase high-performance liquid chromatography; ODS, octadecylsilane; S.D., standard deviation; CV, coefficient of variation; *r*, correlation coefficient.

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fermented foods, beverages, rat tissues, and human serum is described. Application of this method to the serum of possible biotinidase deficiency patients was also presented.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetonitrile, ethanol, and ethyl acetate were of high purity (> 99.8%), D-biotin, D,L-alpha-lipoic acid ((±)-thioctic acid), activated charcoal (acid washed; for column chromatography; P/N 035-18081), propan-2-ol (HPLC grade), ethylene glycol (amino acid analysis grade), 25% ammonia water (metal analysis grade), sulphuric acid, sodium chloride, and sodium dihydrogenphosphate dihydrate were purchased from Wako (Osaka, Japan). D-Desthiobiotin (5-methyl-2-oxo-4-imidazolidine hexanoic acid; D 1411), biocytin (Bct; ε-*N*-biotinyl-L-lysine, Mr 372.5; B 4261), and biotin methyl ester (B 7883) were from Sigma (St. Louis, MO, USA). 9-Anthryldiazomethane (ADAM) was from Funakoshi Pharmaceutical (Tokyo, Japan). A 0.25% (w/v) trypsin-EDTA solution was purchased from Invitrogen Co. (Grand Island, NY, USA).

A light-intercepting micro tube with a cap (2 mL; P/N 72.693.018) and a microtube with cap (2 mL; P/N 72.694.007) were obtained from Sarstedt Aktiengesellschaft & Co. (Nümbrecht, Germany). Microcentrifuge tubes (1.5 mL, polypropylene, lock-cap; P/N 96.8668.9.01) were from Treff AG, Degersheim, Switzerland. Membrane filters of Ekicrodisc 13 CR (0.2 μm; PTFE; P/N E135), Ekicrodisc 13 (0.2 μm; Versapor; P/N E134), and Ekicrodisc 25 (0.2 μm; Versapor; P/N E254) were from Pall Japan Co. (Tokyo, Japan). pH indicator papers (pH 6.4–8.0, narrow range) were from Whatman, Maidstone, Kent, England. Blades and disposable scalpels were from Feather Safety Razor Co., Osaka, Japan.

The affinity gel was chicken egg-white avidin (Bioptic AV-1, 5 μm diameter silica gel) was from GL Sciences Inc. Tokyo, Japan. Affinity gel, 5 g wet weight, was suspended in phosphate-buffered saline (PBS; 2.5 mL): 5 mL trypsin-EDTA solution was added, and incubated at 37 °C for 3 min. This was centrifuged at 3000 rpm for 3 min, and the supernatant was discarded. After adding 10 mL of PBS, the gels were similarly centrifuged. This washing by centrifugation was repeated for 6 times. Two lots of trypsin-treated avidin gels were prepared. The trypsin-treated gel was then packed into the 33 mm × 4.6 mm I.D. stainless steel column (Column no.1 using lot 1 gel), and eluted with solvent B (flow rate; 1.0 mL/min) at 50 °C for 1 h in order to elute the remaining trypsin and peptides, and to equilibrate the gel to the acidic eluent. Three other columns (Column no. 2; 33 × 4.0 mm I.D. with lot 1 gel, Column no. 3; 33 × 4.6 mm I.D. with lot 2 gel, and a Column for lipoic acid analysis; 20 × 4.0 mm I.D. with lot 1 gel) were prepared similarly. As a reference an intact avidin column (33 × 4.0 mm I.D.; intact gel of lot 2) was also prepared.

The commercially available nutritional supplements "Nature Made Multiple Vitamin" (indicated biotin content; 31.3 μg/g dry weight) and "Multivitamin & Mineral" (indicated biotin content; 7.5 μg/g dry weight) were purchased from Otsuka Pharmaceutical Co., Tokyo, Japan, and Kyowa Fermentation Technology Co., Tokyo, Japan, respectively.

Six kinds of "nattos" (Japanese fermented soybean food; such as kizami-natto, from ground (1/15) soybeans, Yamada Foods Co., Misato-cho, Senboku-gun, Akita, Japan), three sakes (rice wine; such as Souka, Horan Brewery, Otawara-city, Tochigi, Japan), beers (Kirin Ichiban-Shibori; Kirin Beer Brewery Co., Tokyo, Japan, and Heineken; Heineken Japan Co., Tokyo, Japan), coffee, red wine, anchovies fillets in olive oil, bread, vinegar (komedzu made from sake, and balsamico made from wine), banana, sauerkraut (Hengstenberg, Esslingen, Germany), "shiitake"(mushroom), soy sauce (Kikkoman Co., Noda City, Chiba, Japan), "miso" (soybean paste), chicken eggs, "sujiko" (salmon roe), sea urchin roe, black pepper, garlic, rice bran, wheat flour (strong and weak), buckwheat flour, potato flour, bovine milk (purchased in February (winter) and May (summer)), Yakult beverage (purchased in February and May, Yakult Co., Tokyo, Japan), peanut (parched), soybeans (parched), pickles, sesame oil, rice bran oil, "nukamiso-zuke" (vegetables pickled in fermented rice bran, Lactobacillus, and yeast), "tofu" (bean curd), honey, "komatsuna" (*Brassica rapa* var. *pervidis*), spinach, pork (thigh), "mekabu" (the sporophylls of seaweed "wakame" (*Undaria pinnatifida*), "kombu" (tangle; *Laminaria japonica*), "nori" (laver; *Porphyra tenera*), bamboo shoot, and chocolate were purchased from grocery stores. Dried yeast (The Japan Pharmacopoeia; Ebios; Tanabe Pharmaceutical Co., Osaka, Japan) was purchased from a drugstore. Royal jelly was purchased from the apiary (San Ken Co., Tokyo, Japan). A flower of anemone (*Anemone coronaria*) was purchased from a flower shop.

Human serum was kindly donated by volunteers. LEW rats (9 weeks of age; male) were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan). Human breast milk (9 months after parturition) and human urine were supplied by healthy volunteers.

Lactobacillus casei (*Shirota*) was prepared as follows. The winter Yakult beverage (0.05 mL) was centrifuged at 12,100 × *g* for 3 min, and the supernatant fraction and precipitated bacterial cells were collected. Bacterial cells were resuspended in 0.05 mL distilled water.

Bacillus natto was prepared as follows. Natto (100 g wet weight, 150% natto kinase strain, Yamada Foods Co., Ushiku City, Ibaragi, Japan) was mixed with a pair of chopsticks, and suspended in 500 mL PBS. This suspension was filtered through bleached cotton cloth. The filtrate was ultracentrifuged using a rotor type 35 (Beckman L8-M Ultracentrifuge) at 12,000 × *g* for 15 min. The bacterial cells were washed and centrifuged three times with PBS and two times with distilled water at 4 °C. A concentrated aqueous solution of 3.20 g wet weight (0.275 g dry weight) of bacterial cells was obtained.

The bacterial cells were lysed as follows. One millilitre of cell suspension was added 5.5 mg of chicken egg white lysozyme (Wako) and incubated at 37 °C for 3 min. Mucous bacterial cells were then ultrasonicated for 10 min. The resulted non-mucous solution was used as the bacterial cell lysate to determine the free biotin.

2.2. High-performance liquid chromatography

The HPLC system used was a two-pump gradient system (two LC-10AD pumps with an SCL-10A system controller; Shimadzu, Kyoto, Japan). Eluent was prepared as follows: 2 L of 0.1 M of sodium phosphate buffer (pH 2.0; Mönch's solvent [3]) containing 0.7 M NaCl was prepared. Then 200 mL of this solution was added to 200 mL of ethylene glycol, 600 mL of propan-2-ol and 12 mL of phosphoric acid making 1012 mL of solvent B. Solvent A was prepared by the addition of 200 mL of Solvent B to the remaining 1800 mL of sodium phosphate buffer containing 0.7 M NaCl. The pH of both solvents was 2.4. The initial flow rate was 0.38 mL/min, the initial concentration of solvent B was 15%, and the gradient programme shown in Table 1 was used. The column temperature was 17 °C (column oven with cooler, Model CTO-10 ACvp; Shimadzu), and the column inlet pressure was 4.12–25.4 MPa (613–3788 psi; 43–266 kg/cm^2). A Model U6K injector (Waters, Milford, MA, USA) was used with a 0.1 mL sample loop. Biotin-ADAM was detected with a fluorescence detector (Shimadzu Model RF-10Axl with Cell Temp Controller) at an excitation wavelength of 365 nm and an emission wavelength of 412 nm at a flow-through cell temperature of 20 °C. Parameters used for the fluorescence detector were

Table 1
Typical elution programme for the trypsin-treated avidin-affinity column used for the biotin analysis[a]

Time (min)	Function	Value (%)	Value (mL/min)
0.01	B conc.	15.0	
0.01	T flow		0.38
0.01	B conc.	15.0	
1.0	T flow		0.38
16.0	T flow		0.38
16.01	T flow		1.0
31.99	T flow		1.0
32.0	T flow		0.38
32.0	B conc.	15.0	
32.0	B curv	0	
32.0	T flow		0.38
77.0	T flow		0.38
77.0	B conc.	55.0	
77.01	B conc.	99.0	
77.01	T flow		0.95
84.99	B conc.	99.0	
85.0	T flow		0.95
85.0	B conc.	15.0	
87.98	T flow		0.95
87.99	T flow		0.38
88.0	Stop (of programme)		

[a] A Shimadzu LC-10AD two-pump system (system controller: SCL-10A) was used. The injector was a model U6K (Waters) with a sample-loop of 0.10 mL–internal volume. Column temperature was 17 °C. Initial conditions were flow-rate 0.38 mL/min and 15.0% concentration of solvent B. B conc.: concentration (%) of solvent B. B curve: curve mode, 0 was linear. T flow: flow rate (mL/min). Stop: end of programme. One cycle of analysis takes 97 min. Other conditions are as described in the Section 2.

as follows; i.e., gain 1, sens 1, and range 3 . One analysis cycle took 92 min.

2.3. Determination of biotin in foodstuffs, tissues, and body fluids

Hydrolysis treatment was performed in 0.35 mL of sample plus 0.05 mL of conc. sulphuric acid (final concentration of sulphuric acid at 2.25 M) at 120 °C for 1 h [4] in the light-intercepting micro tube with a cap. Usually, 0.05–0.2 mL of liquid samples, and 5–50 mg of powdered or wet solid samples were hydrolyzed. After hydrolysis, 0.40 mL of 2.25 M NaOH was added to neutralize the hydrolysate. The final pH was adjusted to between pH 5–6 by adding a 0.15 mL of 1.4 M phosphoric acid (10-fold dilution of phosphoric acid) using litmus papers. Then, total volume was made up to 1.2 mL by adding 025 mL of distilled water, and filtered through Ekicrodisc 13 or 25. About 3.5 mg of activated charcoal was then added to this filtrate to bind the liberated biotin to the charcoal. The mixture was centrifuged at 12,000 rpm (12,100 × *g*; Tomy MC-15A, Tomy Seiko Co., Tokyo, Japan) for 3 min. The charcoal was washed three times with distilled water to reduce the salt concentration. The bound biotin was then extracted from the charcoal by adding 1 mL of 5% ammonia ethanol solution (25% ammonia water : ethanol = 9: 36, v/v). The charcoal supernatant was filtered, Ekicrodisc 13CR, and the filtrate evaporated to dryness under nitrogen. The dried extract was reconstituted in 100 μL of methnol and then 80 μL of 0.1% ADAM (w/v in ethyl acetate) was added. The mixture was vortexed and allowed to stand for 1 h at room temperature, wrapped in aluminium foil, and stored at −20 °C until analysis. Before analysis, it was diluted 10- or 100-fold diluted with methanol and filtered again through Ekicrodisc 13CR into the light-intercepting micro tube with a cap (2 mL). A portion (1–5 μL) of the diluted sample was injected into the HPLC system. These diluted samples and the non-diluted original samples were stored for more than a year at −20 °C. This determination process was repeated three times, and the mean value was obtained.

Standard solutions were made as follows; 0.1 mL of 1 mM biotin (in methanol), 1 mM lipoic acid (in methanol), or 1 mM desthiobiotin were mixed with 0.08 mL of 0.1% ADAM solution. The mixture was allowed to stand for 1 h at room temperature wrapped in aluminium foil, and then stored at −20 °C until analysis. Before analysis, the control was diluted 1000- or 20,000-fold with methanol, and a portion (1–5 μL) of the diluted standard was injected into the HPLC system.

Free-form biotin of cell lysate of *Bacillus natto*, "the multiple vitamin", "multivitamin and mineral" supplements, and human serum were measured as follows. The samples were suspended in 85–95% methanol, and ultrasonicated for 5–20 min. After filtration through Ekicrodisc 13CR or 25, the filtrate was dried under a stream of nitrogen gas. The dried extract was dissolved in 1 mL of distilled water and 0.015 mL of 2.25 M NaOH. After 0.06 mL of 1.4 M phosphoric acid (pH ca. 5.4) was added and mixed, activated charcoal (ca. 4 mg) was added. Then charcoal was washed by centrifugation with PBS 3 times and with distilled water three times. Then, bound free-biotin was extracted with 5% ammonia-ethanol, and the extracted biotin was derivatized by ADAM as above.

2.4. Recovery tests

Recovery of biotin from Bct was determined as follows. Bct was dissolved in distilled water at 100 μM for the stock solution. For 10 μM Bct, 0.01 mL of 100 μM Bct, 0.0775 mL of distilled water, and 0.0125 mL of conc. sulphuric acid were mixed (final sulphuric acid concentration 2.25 M), and hydrolyzed at 120 °C for 1 h. Similar mixing procedure was done for 5 μM Bct. Liberated biotin was collected by the charcoal as described above, and processed. The ratio of found amount of biotin and directly derivatized biotin-ADAM (expected biotin) was calculated and expressed as total-biotin recovery (Table 2; upper table).

Recovery of biotin added to 0.05 mL of healthy human serum (adult female) was also determined as follows. Biotin (0.122 or 0.061 μg) was added to 0.05 mL of human serum to a concentration of 10 and 5 μM, respectively. The biotin concentration was determined as above, and the correction of 1.52 was performed. These

Table 2
Recovery tests of biotin from biocytin (Bct) and of added biotin from human serum[a]

Recovery of biotin from biocytin (Bct)[b]		
Bct concentration (μM)	Mean recovery ± S.D. (%)	CV (%)
10	65.1 ± 2.03	3.14
5.0	67.3 ± 2.45	3.64
Average of recovery measured at 10 and 5.0 μM (n = 18)		
	65.7 ± 2.53	3.48

Recovery of added biotin from human serum (total-biotin method)[c]			
Added concentration (μg/mL)	Found (μg/mL)	Recovery ± S.D. (%)	CV (%)
2.44	2.39	97.5 ± 6.72	6.89
1.22	1.22	100 ± 17.0	17.0
Recovery of added biotin from human serum (free-biotin method)[d]			
Added amount (μg)	Found (μg)	Recovery ± S.D. (%)	CV (%)
0.488	0.350	70.7 ± 10.9	15.4

[a] Recovery test was performed nine-times as described in the Section 2. SD; standard deviation, CV; coefficient of variation = (S.D./mean) × 100 (%).

[b] Using this recovery value (65.7%), the correction factor for total biotin assay was estimated as 1.52 (=1/0.657).

[c] 0.05 mL of the human serum from an adult healthy volunteer (female) was used for the recovery test for total biotin (n = 3). Total biotin of the serum was 1.56 μg/mL. Correction factor of 1.52 was used.

[d] 0.20 mL of the human serum from an adult healthy volunteer (female) was used for the recovery test for free biotin (n = 3). Using this recovery value (70.7%), the correction factor for free biotin assay was estimated as 1.41 (=1/0.707). Free biotin of the serum was 0.0495 μg/mL.

spiked serum samples were compared to the original serum (no addition control) (Table 2: upper bottom table).

Recovery of free biotin added to 0.2 mL of healthy human serum (adult female) was also determined as follows. Biotin (0.488 μg) was added to 0.2 mL of human serum to a concentration of 10 μM. The free biotin concentration was determined as above, and no correction was performed. These spiked serum samples were compared to the original serum (no addition control) (Table 2; lower bottom table).

2.5. Statistics

Non-parametric statistical analysis was performed according to ref. [5]. Significance of the correlation coefficient (r) of the calibration line through the origin was obtained as $p < 0.01$ [6]; i.e., the quantity (in pmol) of injected biotin and peak area (arbitrary unit) obtained, respectively, were 1.39 and 1.32, 1.11 and 1.07, 0.555 and 0.498, 0.0695 and 0.058, 0 and 0 (sample number (N) = 5, and degrees of freedom $n = N - 2 = 3$).

3. Results and discussion

The commercially available avidin affinity column (25 cm long) is too long to analyze the biotin derivative; i.e., avidin binds biotin too strongly to give a tailed biotin-ADAM peak. Previously, a similar strong affinity phenomenon of proteins to the ODS-bound silica gel column (RP-HPLC) was observed [7]. However, the use of an acidic alcoholic (propan-2-ol) eluent (pH 2.1), a low temperature (15 °C), and a short column length (5 cm long) for the protein analysis was found to be successful [7]. Similarly, a short column (3.3 cm long) successfully yielded a good peak shape for the strongly binding biotin-ADAM using the acidic alcohol eluent (Fig. 1; lower panel). However, this intact avidin column was found to be unable to separate the biotin peak from other interfering peaks in this acidic alcohol eluent (Fig. 1; lower panel), i.e., a wider peak and a slight reduction in the retention time were usually observed during searching the separation condition. During the study, we observed that the trypsin-treated avidin gel yielded a good peak shape for the 3.3 cm column, and which was applicable to serum samples (Fig. 1; upper panel). The chromatograms of standards using the gradient programme of Table 1 with trypsin-treated column no. 1 are shown in Fig. 2. A 20 × 4.0 mm I.D. column showed a good peak shape for lipoic acid, but gave a broad peak for biotin (data not shown). Therefore, 33 mm column was found to be suitable to biotin analysis, on the other hand the 20 mm column was optimal for the lipoic acid analysis. As shown in Fig. 2, desthiobiotin (retention time 54 min; top chromatogram) eluted before the biotin peak (retention time 58 min). The ability of this column to separate biotin and desthiobiotin is also shown in the third chromatogram from the top. A linear calibration line through the origin was obtained between 0 and 1.39 pmol of biotin ($r = 0.9997$ ($p < 0.01$): $y = 0.959 \times -0.005$, correlation between injected biotin-ADAM (pmol) and corrected peak area), and the detection limit was 69.5 fmol (S/N = 3; data not shown). Peak area was measured by weighing the peak area manually.

The result of the linear calibration line through the origin seemed to be due to the inclusion of sodium chloride (0.7 M) in the eluent, because we also observed a similar effect of sodium chloride on the calibration line [2]. Inclusion of sodium chloride in the eluent may be good for separations involving proteins [2,8]; i.e., dipole–dipole interactions of two polar molecules between eluting biotin molecule or protein and avidin protein in the stationary phase may become ideal through the Debye shielding effect by high concentrations of sodium ions. In this context, use of lithium ions instead of sodium ions may be more effective [9].

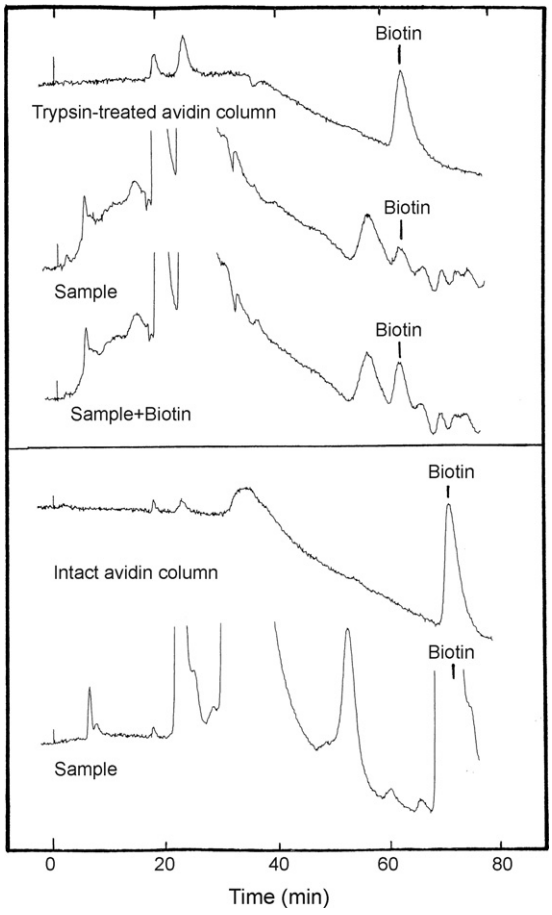

Fig. 1. Effect of the trypsin treatment to avidin-bound gel on the serum total biotin analysis. The same time programme of Table 1 (except from 15% of solvent B to 42% of solvent B at 77 min) was used. Upper panel; trypsin-treated avidin column (no. 3; lot 2, 33 mm × 4.6 mm I.D). Serum total biotin was readily measurable soon after the column preparation. Upper chromatograms in each panels were standard biotin (1.67 pmol). Sample of the upper panel; 0.001 mL of the total biotin sample (10-fold diluted) prepared from the serum of a primary biliary cirrhosis patient (adult female). Total biotin was determined as 4.56 μg/mL. Sample + biotin; 0.001 mL of the total biotin sample (10-fold diluted and 0.557 pmol of the standard biotin were injected simultaneously into the HPLC system lower panel; intact avidin column (intact gel of lot 2). Sample of the lower panel; 0.001 mL of the free biotin sample (10-fold diluted) prepared from the suspected biotinidase deficiency patient (4 months, male). Serum free-biotin was not able to be measurable after the intact avidin column preparation. This patient's free biotin was determined separately as 239 ng/mL using trypsin-treated avidin columns. Retention times of biotin-ADAM were 70.4 min for intact avidin column and 61.2 min for trypsin-treated avidin column, respectively. Therefore, retention time of biotin of the trypsin-treated avidin column was 13% smaller than that of the intact avidin column.

A gradient programme was devised to elute the non-reacted ADAM reagent first (from 16 to 32 min, at 1.0 mL/min), then a final column washing with a high concentration of propan-2-ol (from 77 to 85 min, 0.95 mL/min) (Table 1). The first thorough washing procedure from 16 to 32 min may be a unique characteristic to the affinity column, since the retention time of the biotin-ADAM depended only on the increment of the concentration of propan-2-ol, and was not affected by this first washing procedure at the initial constant propan-2-ol concentration at all. The final column washing with a high concentration of propan-2-ol (from 77 to 85 min, 0.95 mL/min) was also essential for repeatability and reproducibility; i.e., to clean up thoroughly the biotin-binding portion of avidin was essential. This finding of necessity of thorough washing with high concentration of alcohol may be general for var-

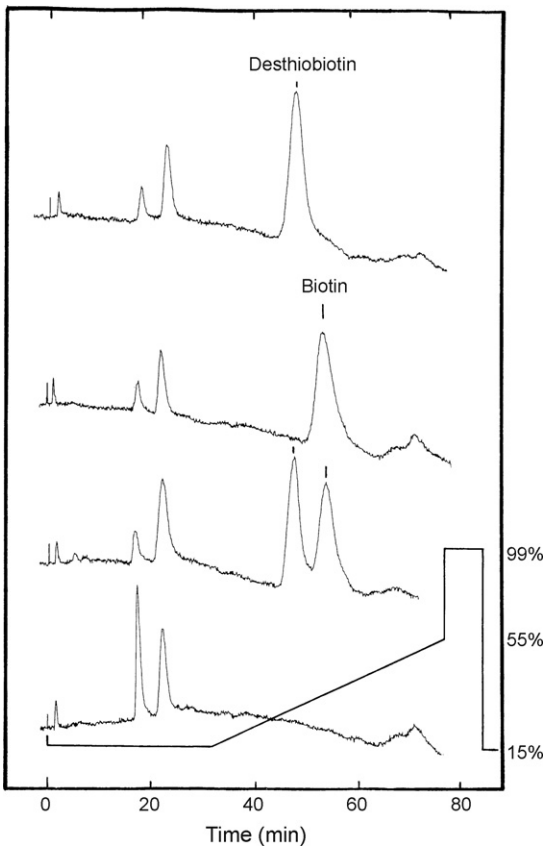

Fig. 2. Separation of standard biotin-ADAM and desthiobiotin-ADAM. Top and second from the top chromatograms: 1.39 pmol of desthiobiotin and biotin. Desthiobiotin or biotin (0.10 mL of 1 mM solution) was reacted with 0.08 ml of 0.1% ADAM, and 0.005 mL of 2000-fold dilution with methanol of standards was injected. The third chromatogram from the top: 0.005 mL of a mixture of standards (1000-fold diluted standards were mixed (50:50 (v/v))) was injected. Lower chromatogram: reagent blank. For the blank, the linear gradient programme (Table 1) of increment of solvent B (%) was also shown. Other conditions were as described in the Section 2.

ious binding phenomena for many binding proteins, such as avidin, immunoglobulins, and other hormone-binding proteins.

Desthiobiotin was found in the biotin-producing plants and microbes, but animal samples showed no indication of the presence of desthiobiotin.

Recovery tests of this procedure were performed, and the results are summarized in Table 2 (top table). The recovery values of biotin from 10 and 5.0 μM Bct were similar, and a mean recovery of 65.7 ± 2.53% (CV = 3.48%) was obtained for this analytical method. The added amount of activated charcoal seemed not to be critical for biotin analysis (data not shown). The repeatability (n = 3) of this method on liquid and solid samples was examined. Heineken beer (liquid) was 0.223 ± 0.0173 μg/mL (CV 7.75%), perfused rat liver (solid) was 6.55 ± 0.251 μg/g wet weight (CV 3.83%), and mouse liver (solid) was 7.46 ± 0.418 μg/g wet weight (CV 5.60%), respectively, and CV values within 8% were observed. Further, recovery of biotin added to healthy human serum was also measured. As shown in Table 2 (upper bottom table), recovery values using correction factor 1.52 were more than 98% (total-biotin method). This result supports the validity of this method for clinical serum sample.

Therefore, the average of three determinations seemed to be sufficient to perform the total biotin determination, and the correction factor of 1.52 (= 1/0.657) was used in the following tests.

Furthermore, recovery of free-form biotin added to healthy human serum without acid hydrolysis was also measured (Table 2, lower bottom table). This free-biotin method (extraction by 95% methanol) gave a recovery value of 70.7 ± 10.9% (CV = 15.4%). Our previous paper also indicated that free biotin determinations using ODS column showed a relatively high CV values of 17.6% (at 406 μM) and 27.5% (at 4.06 μM), respectively (Table 1 of ref. [1]). The binding of free form biotin to the activated charcoal may be strongly interfered by many hydrophobic compounds of the serum, which gives a relatively high CV values as compared to the total method. For the free biotin determination, the correction factor of 1.41 (= 1/0.707) was used in the following tests.

Commercially available nutritional supplements with the indicated biotin concentrations of 31.3 μg/g and 7.5 μg/g dry weight were then tested. The fee biotin of the multiple vitamin was 31.9 μg/g (1.9% higher than the indicated biotin value), and that of the multivitamin and minerals was 8.07 μg/g (7.6% higher than the indicated biotin value), respectively. These results also indicate that this method is applicable to nutritional supplements.

In Table 3, biotin concentrations of representative foodstuffs, tissue and body fluids are shown in decreasing order. The biotin concentration was the highest in chicken egg yolk, natto (a Japanese soybean food fermented by *Bacillus natto*) and abundant in rice bran, royal jelly, dried yeast (Ebios), pepper, coffee, peanut, kidney, sujiko, bread, and liver (Table 3). Biotin was first purified from chicken egg yolk by Kögl and Tönnis [10]. Among the nattos examined, the biotin concentration of kizami-natto made from ground (1/15) soybeans was the highest at 49.7 μg/g of wet weight, and that of ordinary natto made from large soybeans was the lowest at 10.0 μg/g of wet weight; thus, the biotin concentration depended on the surface area of soybeans and the strain of *Bacillus natto*.

In the animal tissues, the biotin concentration was high in kidney and liver and scarce in porcine thigh muscle (Table 3). The biotin concentration of bovine milk in summer (May) was about 3- or 4-fold higher than that in winter (February) (Table 3). A similar seasonal difference in biotin concentration in bovine milk has already been reported by Suzuki using bioassay using rats (table on page 593 of ref. [11]). He was the first to suggest that biotin (vitamin H) was present in great quantity in rice bran (Table 3, and table on page 593 of ref. [11]). Biotin content in royal jelly was also high as in Table 3, and it was found that the pollen from a flower of anemone also contained high total biotin (12.0 μg/g wet weight).

The Yakult beverage (containing *Lactobacillus casei (Shirota)*) is a fermentation product of bovine milk, and it showed similar seasonal differences in biotin concentrations similar to that of bovine milk (Table 3). The 87% of biotin of the winter Yakult was present in the bacterial cells (at 0.16 μg/mL), however, only 13% (0.025 μg/mL) was present in the supernatant fluid. Therefore, the biotinidase of *Lactobacillus casei* (*Shirota*) [5] may concentrate the biotinyl compounds from bovine milk into the bacterial cells.

Therefore, biotin was considered to be highly synthesized by the fermentations by *Bacillus natto*, yeast, and *Acetobacter aceti*, except the fermentation by *Lactobacillus casei*. Systems for the next generation, such as chicken egg yolk, royal jelly, pollen, fish eggs, cereal seeds, beans, banana, and the sporophylls of seaweed wakame (*Undaria pinnatifida*) also contained abundant biotin (Table 3). In this regard, it is interesting that testis and ovary are the highest tissues next to kidney with respect to biotinidase specific activity (*V*) in LEW rat [5].

The biotin content of porcine chop was reported to be 0.045 μg/g [12]. Our value for ham was 0.083 μg/g (Table 3), and the smaller value for porcine chop in ref. [12] may be due to the difficulty in handling avidin, i.e., thorough washing of avidin was very difficult as described above. Further, our attempt to hydrolyze Bct by the procedure in ref. [12] (at 2.25 M HCl, 100 °C for 2 h) was unsuccessful to liberate biotin (data not shown). Our value for winter milk was 0.16 μg/mL (Table 4) and that of whole milk of unknown season

Table 3
Biotin content in the foodstuffs, tissue and body fluids in decreasing order[a]

Samples	Biotin		
	(nmol/g or [b]nmol/mL)	(μg/g or [b]μg/mL)	(μg/g)[c]
Chicken egg yolk[d]	144	35.2	
Natto (n = 6)	97.6 (41.0–204)	23.8 (10.0–49.7)	
Rice bran[d]	84.3	20.7	[e]
Royal jelly[d]	84.3	20.6	
Dried yeast (Ebios)[d]	63.5	15.5	
Pepper[d]	57.3	14.0	
Coffee (parched, ground)[d]	55.3	13.5	
Peanut (parched)[d]	52.9	12.9	
Kidney (rat)	23.1	5.64	3.9 (Bovine), 7.8 (Porcine)
"Sujiko" (salmon roe)	18.1	4.42	
Bread	14.5	3.54	
Liver (rat)	13.6	3.32	3.9 (Bovine, Porcine)
Mekabu (seaweed)	12.4	3.03	
Vinegar (from sake)	9.54	2.33	
Soy sauce	9.34	2.28	
Garlic	8.96	2.19	
Sauerkraut	8.80	2.15	
Buckwheat flour[d]	8.19	2.00	
Beer (1st pressed malt)	8.13	1.99	
Potato flour[d]	7.99	1.95	0.62–0.96
Human serum (Healthy) (n = 3)	7.38 (6.89–7.89)	1.80 (1.68–1.93)	
Wine (red)	5.56	1.36	
Wheat flour (strong)[d]	5.22	1.28	
Banana (n = 3)	4.0 (2.1–5.2)	0.99 (0.51–1.27)	0.39–0.48
Anchovy in olive oil	3.9	0.94	
Yakult beverage (summer)	3.8	0.92	
Yakult beverage (winter)	0.76	0.19	
Chocolate (solid)	3.0	0.74	
Bovine milk (summer)	2.6	0.62	0.20–0.39 (summer)
Bovine milk (winter)	0.68	0.16	0.098 (winter)
Human urine	1.7	0.43	
Human breast milk	1.5	0.38	
Sake (rice wine) (n = 3)	1.4 (1.1–1.7)	0.34 (0.27–0.42)	
Shiitake (mushroom)	1.0	0.25	
Honey	0.55	0.14	
Leg muscle (porine)	0.34	0.083	0
Spinach	0.21	0.052	
Chicken egg white	ND	ND	
Rice (polished)	ND	ND	0

[a] Correction by the recovery (factor = 1.52) was performed. Other conditions were as described in the Section 2. ND, not detectable.
[b] Liquid samples are expressed as nmol/mL or μg/mL.
[c] As a reference, the corrected and estimated data of Suzuki [11] is also presented.
[d] Expressed by per g dry weight. Other solid samples were expressed by per g wet weight.
[e] It says in ref. [11] that biotin is present in a great quantity.

Table 4
Biotin concentrations (total form and free form biotin) of human serum of possible biotinidase deficiency patients[a]

Serum samples	Total biotin (μg/mL)	Free biotin (ng/mL)	Ratio of free biotin (%)
Patient 1[b] (alopecia, organic acid urea)			
Before biotin treatment	2.03	35.5	1.75
During biotin treatment (1 week)	5.46	154	2.82
During biotin treatment (3 weeks)	4.47	266	5.95
During biotin treatment (7 weeks)	3.05	118	3.87
Healthy baby (1 y, female)	3.21	180	5.61
Patient 2[b](Optical atrophy, polyneuropathy(sensory dominant))			
Before Ebios treatment	1.14	40.6	3.56
After Ebios treatment (4 months)	2.04	77.0	3.77
Near healthy sister (22 y)	1.29	71.0	5.50
Healthy mother (52 y)	1.68	ND	ND
Healthy male volunteer	1.80	122	6.78
Patient 3[b] (Spinocerebellar degeneration, optical atrophy)			
	1.32	110	8.33
Near healthy son (20 y)	1.97	43.9	2.22
Near healthy daughter (17 y)	2.13	103	4.86

[a] Corrections by recoveries were performed. Total: bound- and free-form. Other conditions were as described in the Section 2. ND, not yet determined.
[b] Patient 1: 1 y, female. Patient 2: 32 y, female. Patient 3: 46 y, male.

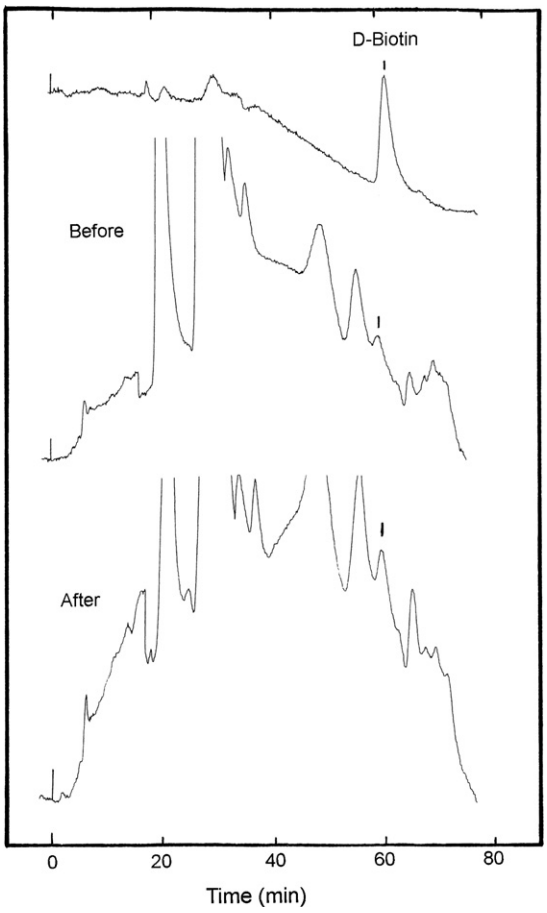

Fig. 3. Direct demonstration of the increase of serum biotin after biotin therapy. A patient doubtful of biotinidase deficiency (1 y, female) was taken biotin (10–15 mg/day); i.e., biotinidase specific activity (V = 118 pmol/min per mg of serum protein) and serum biotin (total; 2.03 μg/mL of serum) were normal, but enzyme seemed to be labile (V was decreased for 18% after heat treatment for 4 h at 37 °C). Affinity chromatographic separation was performed by using the 33 × 4.6 mm I.D. affinity column. From the top, the first chromatogram shows the result of elution of 1.67 pmol of D-biotin (area was 21.6 mg of an A4 paper). The middle chromatogram shows the result of serum analysis of the serum before the biotin taking (biotin peak area was 3.2 mg), and the bottom chromatogram shows the result of analysis of the serum after the biotin taking for one week (biotin peak area was 9.1 mg). A 0.05 mL of serum was used. Reaction mixture was 0.18 mL (180 μL), and 1.5 μL of 10-fold diluted mixture was analyzed. Therefore, 21.6/1.67 = 3.2/X for the serum of before treatment. X equals to 0.2474 pmol of biotin. Then, biotin in 0.05 mL of serum is 0.2474 × 180/(1.5/10) = 297 pmol. And (297/0.05)/1000 = 5.94 nmol/mL. Correction factor for biotin recovery was 1.52, and corrected value becomes 5.94 × 1.52 = 9.03 (nmol/mL). M_r of biotin is 244.31, and the serum biotin content becomes to (9.03 × 244.31)/1000 = 2.20 (μg/mL). Similarly, the biotin content of the serum after biotin treatment is 6.29 μg/mL. Therefore, 2.9-fold increase of serum total biotin was directly demonstrated after the biotin therapy. Other conditions were the same as described in the Section 2. This patient was improved the urine organic acid compositions and alopecia, and left this hospital after 3 weeks of biotin therapy.

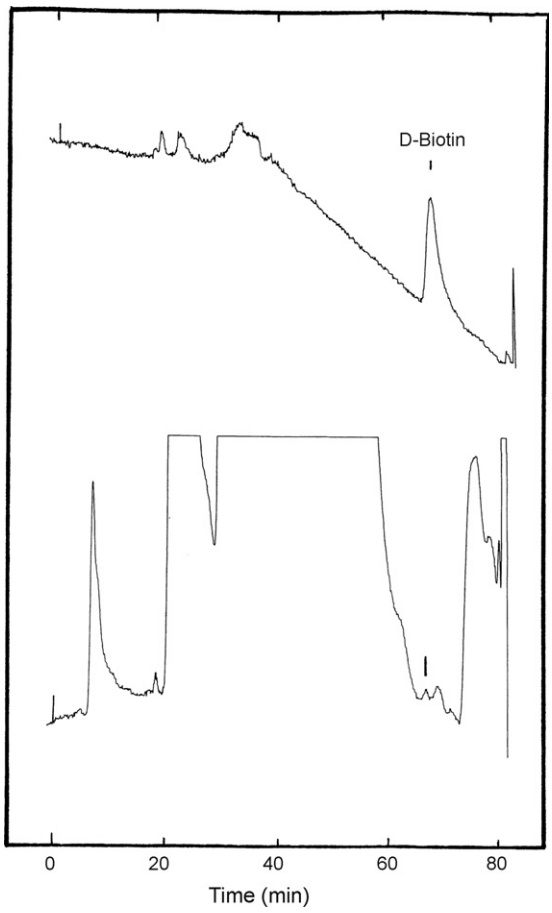

Fig. 4. Typical chromatogram of free biotin analysis of a serum sample. Upper chromatogram: standard biotin (1.67 pmol). Lower chromatogram: a serum sample from a 32 y female of possible biotinidase deficiency (before a medical treatment). 0.2 mL of serum was extracted by the 95% methanol. 0.003 mL of the free biotin sample (10-fold diluted) was injected. The free biotin concentration of this sample was calculated to be 40.6 ng/mL. In order to separate biotin in the case of free form analysis, concentration of the solvent B was reduced, i.e., retention time of the biotin peak in this case was 64.8 min. Other conditions were as described in the Section 2.

was 0.031 μg/mL [13]. This 5-fold smaller value of whole milk by bioassay may be due to a problem with the turbidity measurement in the bioassay, which has a calibration curve with narrow linear region without intercepting the origin. On the other hand, we used a linear calibration line through the origin, i.e., we performed the high recovery analysis [3,7].

Direct demonstration of biotin therapy on a possible biotinidase deficiency patient (1 y, female) was shown in Fig. 3. A 2.9-fold increase of serum total-biotin was directly demonstrated after the biotin therapy (10–15 mg/day) (Fig. 3).

Serum free-form biotin was measurable using the correction factor of 1.41 of Table 2 (Fig. 4). The results of human serum free-form biotin of possible biotinidase deficiency patients together with the results of total biotin were summarized in Table 4. Free biotin concentration of patient 1 (1 y, female) seemed to be low, which was improved by the biotin therapy (Table 4). Increase of total biotin by the biotin therapy of patient 1 was also shown in Fig. 3. Total biotin concentration of patient 2 (32 y, female; showing optical atrophy) was low, which was improved by Ebios treatment. Ratio of free biotin concentration was high in the patient 3 (46 y, male; showing spinocerebellar degeneration) (Table 4). In our previous paper [1], we presented the human serum free-biotin concentration as 21.6 ng/mL (range; 5.4–64.8 ng/mL). The previously reported values contained many serum from allergy patients [1], and the serum was ultrafiltrated by DIAFLO PM10 (Amicon, Danvers, MA, USA). Therefore, our present free-biotin values obtained from the affinity chromatographic method were considered to be similar to the previously presented values [1] obtained by the reversed-phase chromatographic method.

Ratio of free biotin of representative foodstuffs together with human serum was summarized in Table 5. As a reference, ratio of free lipoic acid was also presented in Table 5 [14]. Free biotin was rich in the natto, egg yolk, milk, sake, beer, and royal jelly (Table 5). Because egg yolk, milk and royal jelly are rich in free form biotin, free biotin may be important for the baby or the next generation.

Table 5
Ratio of free biotin (free biotin/total biotin × 100(%)) of representative foodstuffs and human serum[a]

Samples	Ratio of free biotin (%)	Ratio of free lipoic acid (%)
Natto	92.1	13.9
Chicken egg yolk	90.1	21.4
Sake	75.0	7.2
Human breast milk	55.6	21.1
Bovine milk (winter)	40.8	17.1
Yakult beverage (winter)	12.4	1.19
Beer (1st pressed malt)	37.0	4.64
Royal jelly	33.7	1.89
Dried yeast cells (Ebios)	8.3	11.1
Bacillus natto cells	6.9	9.76
Spinach	ND	5.69
Broccoli	ND	4.83
Kmatsuna	ND	2.54
Human serum		
Healthy adult (male)	6.78	2.39
Healthy baby (1 y, female)	5.61	9.17

[a] As a reference, the ratios of free form lipoic acid were also presented. Lipoic acid was determined as in ref. [14]. Other conditions were as described in the Section 2. ND, not determinative due to low biotin concentrations.

Bacillus natto and yeast may be excreting free form biotin in the medium, since ratio of free biotin in natto, sake, and beer is higher than the ratio of free lipoic acid (Table 5). These observations may be of help in the nutritional research.

This affinity chromatographic biotin determination method is rapid, i.e., preparation of sample and analysis was performed within a day for one sample, reproducible, and reliable as compared to the other methods. We expect this method to be a powerful and useful tool in nutritional, food chemical, biochemical, and clinical chemical applications, and for the investigation of diseases related to biotin metabolism.

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