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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