Note

Liquid chromatographic determination of biotin by using 1-pyrenyldiazomethane as a pre-column fluorescent labelling reagent

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Biotin is a coenzyme essential in amino acid or carbohydrate metabolism, and it plays an important rôle in the growth of animals and plants.

Biological methods have generally been used for the determination of biotin[1–4]. However, these methods require culture of the tissue and preservation of its strain. Furthermore, the procedure for sample preparation is tedious and the incubation time-consuming. On the other hand, many workers have reported spectrophotometric methods, based on the binding of a dye to the avidin–biotin complex[5–10], oxidation with potassium iodate[11] or reaction with 4-dimethylaminocinnamaldehyde[12]. However, the selectivities and sensitivities were insufficient for the microdetermination of biotin either in multi-vitamin pharmaceutical preparations or in biological fluids.

Recently, attempts have been made to improve the selectivity by employing high-performance liquid chromatography (HPLC) accompanied by fluorometric detection using 4-bromomethylmethoxycoumarin (Br-Mmc)[13] and 9-anthryldiazomethane (ADAM)[14,15] as the precolumn labelling reagents. However, the use of Br-Mmc involved tedious pretreatment, and the ADAM reagent was unstable.

In the present study, fluorescent labelling of biotin with 1-pyrenyldiazomethane (PDAM), which has recently been developed in our laboratory, was examined. PDAM readily reacted with the carboxylic group of biotin without any catalyst at room temperature, and the ester derivative was separable on a reversed-phase column. The HPLC method was applied to pharmaceutical preparations containing biotin and other vitamins, and to a control serum spiked with biotin.

EXPERIMENTAL

Materials

Biotin was obtained from Sumitomo (Osaka, Japan). Methanol and hexane were HPLC grade (Wako, Osaka, Japan). Other solvents were from Kokusan Kagaku (Tokyo, Japan). All reagents and solvents were of analytical grade. Water was

purified with a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.) before use. A control serum, Consera, was obtained from Nissui Seiyaku (Tokyo, Japan). 1-Pyrenecarbaldehyde was from Aldrich (Milwaukee, WI, U.S.A.). Sep-Pak C_{18} and Si were from Waters Assoc. (Milford, MA, U.S.A.).

A standard solution was prepared by dissolving 100 μg of biotin per 1 ml of methanol and it was diluted in methanol before use.

Derivatization procedure

PDAM was synthesized as described previously[16]. To 100 μl of a sample solution in methanol were added 100 μl of a 1 mg/ml solution of PDAM in ethyl acetate. The mixture was allowed to stand for 60 min at 40°C and then cooled to room temperature. To the reaction mixture were added 800 μl of methanol and an aliquot (5 μl) of the resulting mixture was directly injected into the chromatograph.

On the other hand, an aliquot (50 μl) of the above mixture was injected for normal phase HPLC and the peak fraction of the PDAM derivative of biotin was collected. The fraction was evaporated to dryness and the residue was examined using a mass analyzer Model RKB-9000 (Shimadzu Seisakusho, Kyoto, Japan).

Chromatography

The HPLC system consisted of a Model TRIROTAR-VI (Japan Spectroscopic, Tokyo, Japan) with a degasser Model DG-3510 (Japan Spectroscopic) and an ultra-violet spectrophotometer Model UVIDEC-100-VI (Japan Spectroscopic) or a Model F-1000 spectrofluoromonitor (Hitachi, Japan).

The effluent was measured spectrophotometrically at 240 nm or fluorometrically at the excitation and fluorescence wavelengths of 340 and 395 nm.

The chromatographic separations were performed on a normal phase column packed with LiChrosorb Si 60 (particle size 5 μm, Merck) and a reversed-phase column packed with TSK-gel 80 TM (particle size 5 μm; TOSOH, Tokyo, Japan). Both columns were the same size, 150 mm × 4 mm I.D. In the normal phase mode, the separation was carried out at ambient temperature by using hexane–isopropanol–water (80:20:1) as the mobile phase at a constant flow-rate of 1.0 ml/min. In the reversed-phase mode, the biotin derivative was separated on the ODS column kept at 50°C by using water–acetonitrile (43:57) at a constant flow-rate of 1.0 ml/min.

Application to the biotin preparation

Tablets of a biotin preparation (250 μg per tablet) were finely powdered, and then to the powder containing 500 μg of biotin were added 30 ml of methanol. The mixture was extracted on a Branson Model B-521 ultrasonic cleaner for 10 min and then shaken for 10 min. The mixture was then centrifuged at 3000 rpm for 5 min. The supernatant was transferred to an 100-ml volumetric flask. The above extraction procedure was repeated three times and the supernatants were pooled and made up to 100 ml with methanol. To 200 μl of this solution were added 200 μl of 5 mg/ml PDAM solution in ethyl acetate. The mixture was allowed to stand for 60 min at 40°C and then cooled to room temperature. The resulting mixture was applied to Sep-Pak Si. After washing the cartridge column with 5 ml of hexane, it was eluted with 10 ml of methanol and the effluent was evaporated. The residue was redissolved in 1 ml of methanol and a 5-μl aliquot of the mixture was subjected to HPLC as described above.

Application to biotin-spiked serum

To 1 ml of Consera were added 10 ng/ml biotin solution and mixed. To the resulting mixture was added 1 ml of 10% trichloroacetic acid, and then the mixture was centrifuged at 2000 g for 5 min. The supernatant (1.5 ml) was applied to Sep-Pak C_{18}, which had previously been washed with 10 ml of methanol, 10 ml of water and 5 ml of 1% aqueous acetic acid. After washing the Sep-Pak with 10 ml of 1% acetic acid and 1 ml of water, the biotin was eluted with 10 ml of methanol and the effluent was evaporated. The residue was redissolved in 100 μl of methanol on a ultrasonic cleaner. To 100 μl of this solution were added 100 μl of 1 mg/ml PDAM solution and then the mixture was allowed to stand for 60 min at 40°C. After cooling to room temperature, 300 μl of methanol were added and a 10-μl aliquot of the mixture was directly injected in the chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows the reaction of biotin with PDAM. It was carried out in ethyl acetate at room temperature without a catalyst, reached a plateau in 1.5–2 h and was accelerated by heating. Fig. 2 shows the yield of the reaction as a function of the reaction time at room temperature and at 40°C. The derivatization reaction required 120 min at room temperature or 60 min at 40°C for completion, so the latter condition was adopted in the standard procedure.

The PDAM derivative of biotin was purified by HPLC fractionation and subjected to mass spectrometry to comfirm its structure. The mass spectrum showed the molecular ion peak of the biotin ester at m/z 458, and fragment ions at m/z 215 and 232 formed by ester cleavage.

The excitation and fluorescence spectra of the purified ester were approximately the same in any solvent system used as the mobile phase, showing maxima at 340 and 395 nm.

The separation of the biotin ester was examined by reversed-phase HPLC. Fig. 3 shows a chromatogram of the biotin ester on an octadecylsilyl silica gel column eluted with acetonitrile–water. The biotin ester was clearly separated from the degradation products of PDAM, and it was sensitively detected at the sub-pmol level without interferences from contaminants. Since some contaminants moved far more slowly than the biotin ester under these conditions, it took 80 min to complete the chromatography. The time for separation could, however, be shortened to 40 min by eluting the column with acetonitrile alone immediately after the elution of the biotin ester.
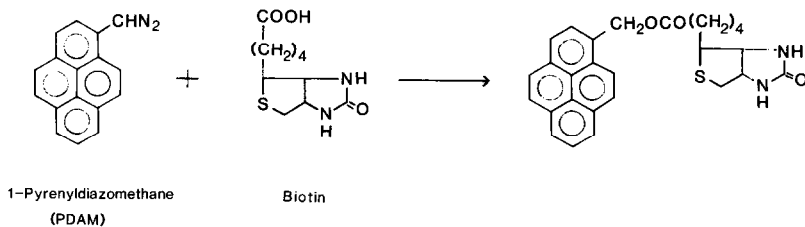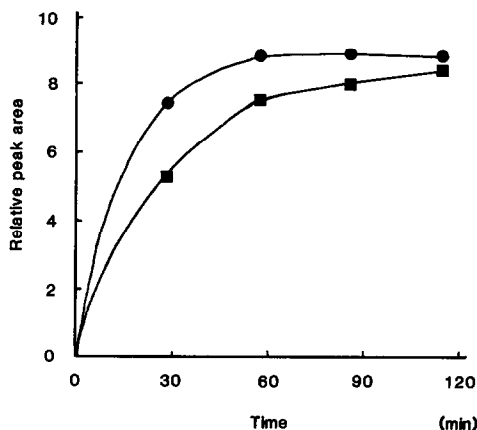

Fig. 1. Reaction course of PDAM with biotin.

Fig. 2. Yield of the PDAM derivative of biotin as a function of reaction time at room temperature (■) and at 40°C (●).

The standard graph for biotin showed excellent linearity in the range from 200 fmol to 200 pmol per injection ($y = 1.101x + 0.022$, $r = 0.999$). The reproducibility of this procedure was also sufficient, the coefficient of variation for 10 pmol of biotin being 1.1% ($n = 5$). The detection limit of biotin under this condition was about 100 fmol per injection (signal-to-noise ratio, $S/N = 3$).

The present standard procedure was applied to the determination of biotin in a vitamin preparation. Both the derivatization and chromatographic separation were not influenced by other components in the preparation. Fig. 4A shows a chromatogram of the PDAM derivative of biotin extracted from the preparation. The recov-
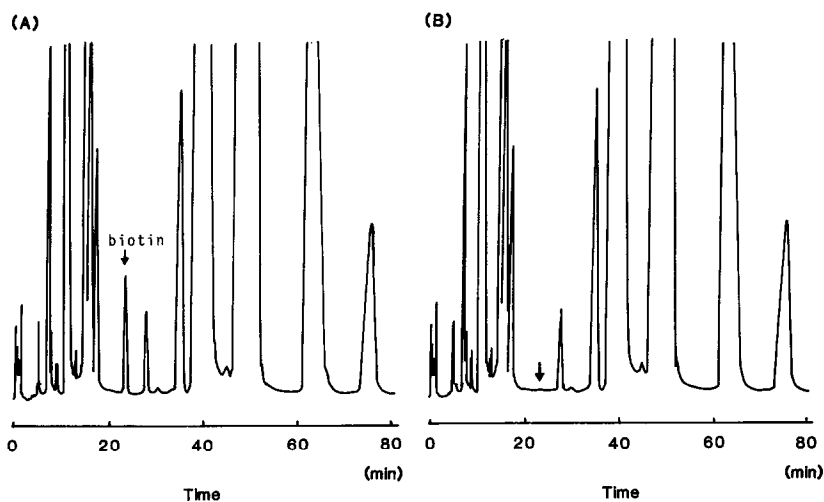

Fig. 3. Reversed-phase liquid chromatographic profiles of the PDAM derivative of standard biotin (A) and a reagent blank (B). Mobile phase, acetonitrile–water (43:57); flow-rate, 1.0 ml/min. The peak corresponds to about 10 pmol of biotin.
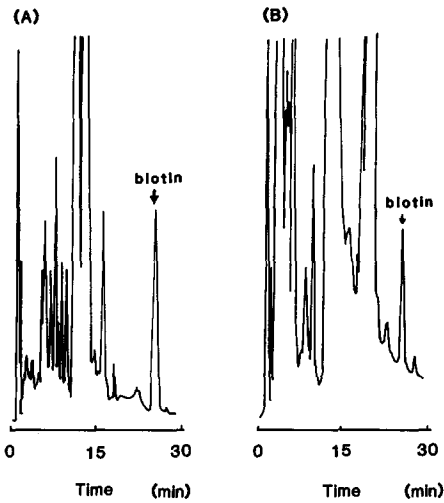
Fig. 4. Reversed-phase liquid chromatographic profiles of the PDAM derivative of biotin extracted from a preparation (A) and from serum (B). Mobile phase as in Fig. 3.

ery of biotin was 98.8% and the coefficient of variation for the reproducibility of this procedure was 1.2% ($n = 4$).

The determination of biotin spiked in serum was also examined by the present method. Extraction of biotin from serum has previously been carried out by using active charcoal[15], but the recovery and reproducibility were poor. We investigated a more efficient extraction method by using a cartridge type extraction column packed with ODS silica gel. The recoveries of biotin from serum at the various concentrations
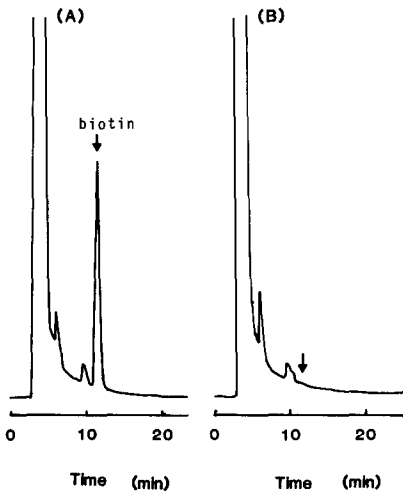

Fig. 5. Normal phase liquid chromatographic profiles of the PDAM derivative of biotin (A) and a reagent blank (B). Mobile phase, hexane–isopropanol–water (80:20:1); flow-rate, 1.0 ml/min. The peak corresponds to about 100 pmol of biotin.

were 90.1 to 104.2%. Fig. 4B shows a chromatogram of the PDAM derivative of biotin extracted from serum.

Furthermore, normal phase HPLC separation of the PDAM derivative of biotin was examined in order to simplify the determination procedure. Fig. 5 shows chromatograms of the derivatization mixture of PDAM and standard biotin, and the reagent blank, directly injected on a silica gel column and eluted with hexane–isopropanol–water (80:20:1). Most of the degradation products of the reagent were eluted at the solvent front, and the PDAM derivative of biotin was clearly separated from these contaminants. However, the sensitivity for the PDAM derivative under this chromatographic condition was about ten times lower than that under reversed-phase conditions, because the relative fluorescence intensities of PDAM derivatives in aqueous media and organic solvents greatly differ. Consequently, the chromatographic conditions should be chosen according to each application.

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