Journal of Chromatography, 542 (1991) 365–372
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 054

High-performance liquid chromatographic determination of biotin in pharmaceutical preparations by post-column fluorescence reaction with thiamine reagent

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(First received July 5th, 1990; revised manuscript received December 6th, 1990)

ABSTRACT

A high-performance liquid chromatographic method for the determination of biotin was devised utilizing a post-column reaction in which the amide bond of biotin was chlorinated and allowed to react with thiamine to give fluorescent thiochrome. A linear relationship was observed between the peak height of biotin in the range 20 ng–3 μg per injection. The limit of detection was 10 ng per injection. The relative standard deviations for 40 and 200 ng per injection of biotin were 3.1 and 1.2% ($n = 10$), respectively. The method could be applied directly to pharmaceutical preparations without any sample pretreatment for the elimination of interfering materials as is required in the conventional methods.

INTRODUCTION

Since biotin was first identified in 1940 by Gyorgy *et al.* [1], many workers have investigated its biochemical functions. The biochemical role of biotin has been revealed to be as the coenzyme of four carboxylases involved in lipid, amino acid, carbohydrate and energy metabolism.

Biotin in biological fluids and pharmaceutical preparations has generally been determined by microbiological methods [2–5], because it has no particular functional group that facilitates sensitive detection, and its contents in such samples are extremely small. Although microbiological methods provide excellent sensitivity, they require long incubation periods and give variable results because they are readily affected by different contaminants. On the other hand, several workers have reported chemical and physico-chemical methods, such as spectrophotometric methods based on the perturbation of dye–protein complexes [6], reaction with 4-dimethylaminocinnamaldehyde [7,8] or oxidation with potassium iodate [9]. However, these methods are neither as sensitive nor as specific as the microbiological methods. Although gas chromatographic methods [10,11] have also been proposed, they require tedious pre-column derivatization and the only method for sensitive detection is mass spectrom-

0021-9673/91/$03.50

etry, which is not readily available in ordinary laboratories. Radiodilution assay [12,13] and radiometric–microbiological assay [14] methods are not as specific and require the manipulation of radioisotopes.

Recently, high-performance liquid chromatographic (HPLC) methods have been applied to the simultaneous determination of various vitamins. As biotin does not show UV absorption suitable for its detection, it was detected either electrochemically [15] or fluorimetrically with precolumn derivatization using reagents such as 4-bromomethyl-7-methoxycumarin (Br-Mmc) [16], 9-anthryldiazomethane (ADAM) [17] and 1-pyrenyldiazomethane (PDAM) [18]. Although highly sensitive and specific, these methods are laborious, because the electrochemical detector requires careful conditioning and the precolumn fluorescence derivatization methods are not easily automated and are prone to interference from contaminants.

We have previously reported [19,20] the fluorimetric assay of proteins in which the amide (peptide) bonds of proteins were chlorinated and allowed to react with thiamine to give fluorescent thiochrome. This paper deals with the application of this principle to the determination of biotin by HPLC.

EXPERIMENTAL

Chemicals

Biotin, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, nicotinamide and folic acid were obtained from Nippon Roche (Tokyo, Japan). Analytical-reagent grade $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot H_2O$, 10% sodium hypochlorite solution (Antiformin), sodium nitrite, sodium hydroxide and Brij-35 and HPLC-grade acetonitrile were obtained from Wako (Osaka, Japan). Sodium 1-butanesulphonate and the tetrapeptide Val–Ala–Ala–Phe were purchased from Tokyo Kasei (Tokyo, Japan) and Sigma (St. Louis, MO, U.S.A.), respectively.

Mobile phase and derivatization reagents

The mobile phase for HPLC was 50 m*M* phosphate buffer (pH 4.5)–acetonitrile (9:1) containing 25 mmol/l of 1-butanesulphonate as the ion-pair reagent. The hypochlorite reagent was prepared by adding 120 ml of 1 *M* sodium hydroxide solution and 4 ml of 25% Brij-35 solution to 80 ml of Antiformin and diluting the resulting mixture with 0.1 *M* phosphate buffer to 1000 ml. The final concentration of available chlorine in the reagent should be 0.8%. Thiamine reagent was prepared by dissolving sodium nitrite and thiamine hydrochloride in 0.1 *M* phosphate buffer (pH 7.5) as described previously [20]. The mobile phase and reagents were filtered through a 0.45-μm microfilter (Fuji Photo Film, Tokyo, Japan) and degassed prior to use.

Chromatographic system

Fig. 1 shows a schematic diagram of the HPLC system. Chromatographic separation was carried out on a 15 cm × 6.0 mm I.D. TSKgel ODS-80TM column (Tosoh, Tokyo, Japan) at 50°C. The mobile phase and the two post-column derivatization reagents were pumped with a Shimadzu LC-6A HPLC solvent-delivery system.

The mobile phase was delivered at a flow-rate of 1.0 ml/min, and 20 μl of the sample solution were injected into the chromatograph using a KMT-60A HPLC

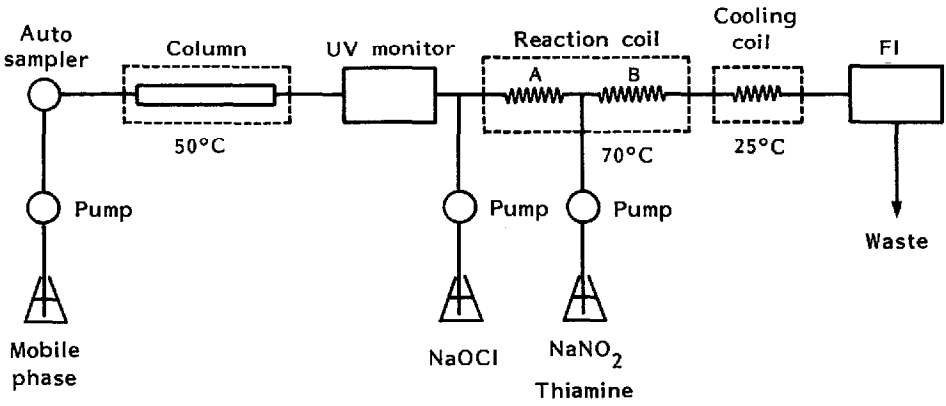

Fig. 1. Schematic diagram of the system for HPLC and fluorescence detection of biotin. Fl = Spectrofluorimeter.

autosampler (Kyowa-Seimitu, Tokyo, Japan). The eluate from the column was first passed through a Shimadzu SPD-6A UV detector equipped with a 20-μl flow cell set at 210 nm, then it was introduced into the fluorescence reactor system. The hypochlorite reagent was delivered to the eluate stream at a flow-rate of 0.3 ml/min and the stream was allowed to flow through the PTFE tubing reaction coil A (3 m × 0.5 mm I.D.) immersed in a water-bath at 70°C (Thermo-minder, Type Ace-80, Taiyo Service Centre, Tokyo, Japan). The eluate was mixed with the thiamine reagent delivered at a flow-rate of 0.3 ml/min, and the resulting mixture was passed through the PTFE tubing reaction coil B (5 m × 0.5 mm I.D.) also in the 70°C water-bath. The effluent from coil B was passed through the PTFE tubing cooling coil (1 m × 0.5 mm I.D.) and its fluorescence intensity was measured at excitation and emission wavelengths of 370 and 440 nm, respectively, using a Shimadzu RF-535 spectrofluorimeter equipped with a 20-μl flow cell.

Analysis of multivitamin tablets and capsules

Twenty tablets and capsules containing biotin were weighed and finely powdered. An accurately weighed portion of the powder equivalent to about 200 μg of biotin was transferred into a centrifuge tube, and 10 ml of the internal standard solution, containing 40 μg/ml of Val–Ala–Ala–Phe in 0.1 *M* Na_2HPO_4, was pipetted into the centrifuging tube. The mixture was shaken for 3 min, ultrasonicated for 20 min and centrifuged at 2000 *g* for 10 min. The supernatant obtained was filtered through a 0.45-μm membrane filter and a 20-μl aliquot of the filtrate was injected into the chromatograph.

Analysis of frozen multivitamin infusion

The multivitamin infusion was determined in the same manner as for tablets and capsules except that ultrasonication and centrifugation were omitted.

RESULTS AND DISCUSSION

Chromatographic separation

Figs. 2 and 3 show the reversed-phase HPLC profiles of a mixture containing biotin and five water-soluble vitamins, *i.e.*, thiamine, riboflavin, pyridoxine, nicotinamide and folic acid, which are usually contained in multivitamin preparations.

The separation was considerably influenced by the pH and composition of the mobile phase. Biotin was well retained on the ODS column in the low pH range, and its peak appeared in the vicinity of riboflavin.

The retention time of biotin decreased with increase in the concentration of acetonitrile in the mobile phase, and it was eluted together with thiamine, pyridoxine, nicotinamide and folic acid. Further, six water-soluble vitamins were not well separated at an acetonitrile concentration of 15% in the mobile phase. When it was decreased to 5%, the biotin was to well retained, giving a retention time over 60 min. Consequently, the pH of the mobile phase was adjusted to 4.5 and the concentration of acetonitrile in the mobile phase to 10%.

In addition to the column used in the standard procedure in the present method, silica-NH_2 and ion-exchange columns were examined for the separation of biotin from the additives in the preparations. However, the former column did not retain biotin and the latter gave poor separations.

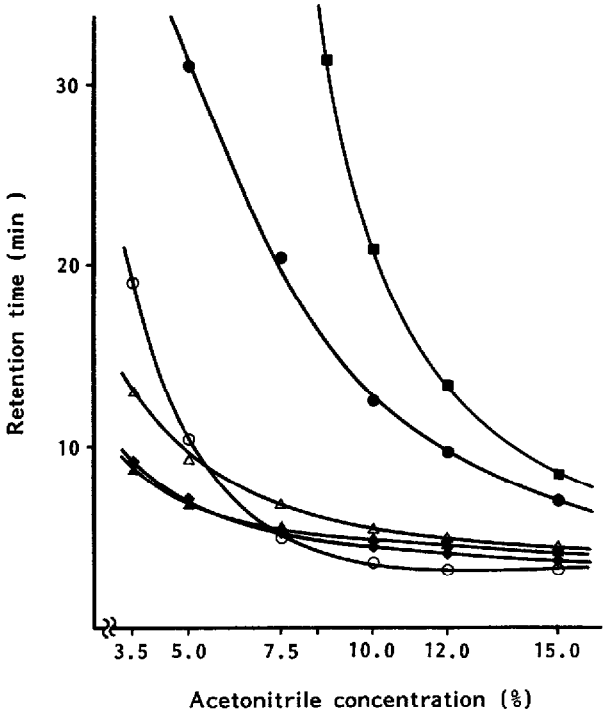

Fig. 2. Retention times of (●) biotin, (△) thiamine hydrochloride, (■) riboflavin, (▲) pyridoxine hydrochloride, (◆) nicotinamide and (○) folic acid plotted against the concentration of acetonitrile in the mobile phase (pH 4.5). HPLC conditions as in the text, except for the acetonitrile concentration.

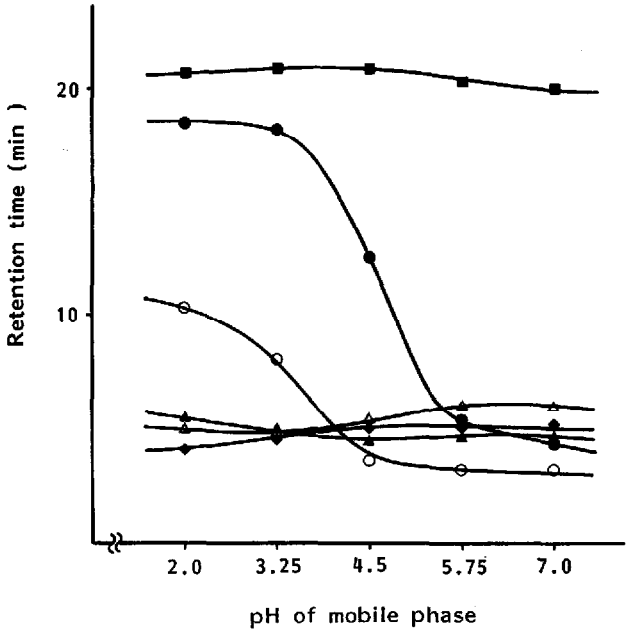

Fig. 3. Retention times of (●) biotin, (△) thiamine hydrochloride, (■) riboflavin, (▲) pyridoxine hydrochloride, (◆) nicotinamide and (○) folic acid plotted against the pH of the buffer in the mobile phase at an acetonitrile concentration of 10%. HPLC conditions as in the text, except for the buffer pH.

Optimization of post-column reaction conditions

The conditions of the reaction were approximately the same as those described previously [20] except for the composition of the hypochlorite reagent. It was necessary to increase the alkalinity of this reagent as the eluate from the chromatograph was acidic whereas the fluorescence reaction requires slightly alkaline conditions. Fig. 4 shows the relationship between the fluorescence intensity and the amount of 1 *M* sodium hydroxide added to the hypochlorite reagent. The maximum fluorescence intensity was observed when 12 ml of 1 *M* sodium hydroxide solution were added to 100 ml of the reagent solution. Acetonitrile or an ion-pair reagent, such as sodium dodecyl sulphate, in the mobile phase did not affect the reaction.

System performance

A linear relationship was observed between the peak height and the amount of biotin injected in the range 20 ng–3 μg, with a correlation coefficient of 0.998. The limit of detection for biotin was 10 ng per injection at a signal-to-noise ratio of 2, which was equal to that of the absorbance at 210 nm, with a relative standard deviations of the fluorescence intensity of 14.1% (n = 10). The relative standard deviations of the fluorescence intensity for 40 and 200 ng per injection of the standard samples of biotin were 3.1 and 1.2% (n = 10), respectively.

Commercial biotin pharmaceutical preparations were assayed utilizing the fluorescence and the absorbance at 210 nm. Fig. 5 shows the chromatograms of several biotin preparations. Table I shows the labelled contents of these preparations. Excel-

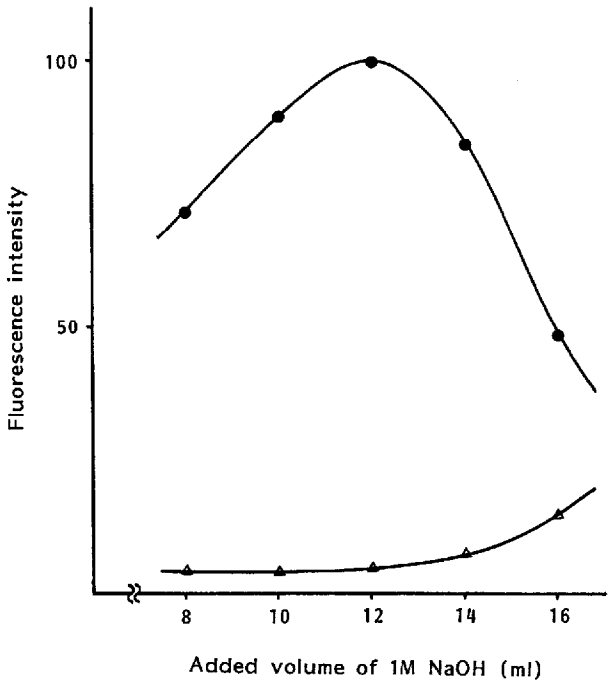

Fig. 4. (●) Fluorescence intensity of biotin (1 μg per injection) and (△) background plotted against the volume of 1 *M* NaOH added to 100 ml of the hypochlorite reagent. HPLC and post-column reaction conditions as in the text, except for the volume of 1 *M* NaOH added.

lent recoveries of biotin were observed with satisfactory reproducibility, as shown in Table II.

As a pretreatment, biotin was extracted from the pharmaceutical products with 0.1 *M* Na_2HPO_4 containing the tetrapeptide Val–Ala–Ala–Phe as an internal standard, and the extract was injected directly into HPLC system. This procedure is much simpler than the methods using Br-Mmc, ADAM or PDAM [16–18].

Absorption at 210 nm, although widely used for detecting substances such as

TABLE I

LABELLED CONTENTS OF BIOTIN IN PHARMACEUTICAL PREPARATIONS

Preparation	Labelled contents
Tablet	Biotin 0.02 mg, bisbenthiamine 8.33 mg, riboflavin 2 mg, pyridoxine · HCl 8.33 mg, nicotinamide 16.67 mg, folic acid 0.13 mg, ascorbic acid 50 mg, cyanocobalamin 0.02 mg, retinol palmitate 666.67 IU, ergocalciferol 66.67 IU, tocopherol calcium succinate 3.46 mg, calcium carbonate 50 mg per tablet
Capsule	Biotin 0.01 mg, L-cysteine 30 mg, riboflavin 15 mg, pyridoxine · HCl 50 mg, nicotinamide 25 mg, calcium pantothenate 15 mg per capsule
Frozen infusion	Biotin 0.06 mg, thiamine · HCl 3.9 mg, riboflavin · Na 4.6 mg, pyridoxine · HCl 4.9 mg, nicotinamide 40 mg, folic acid 0.4 mg, pantothenol 14 mg, ascorbic acid 100 mg per vial

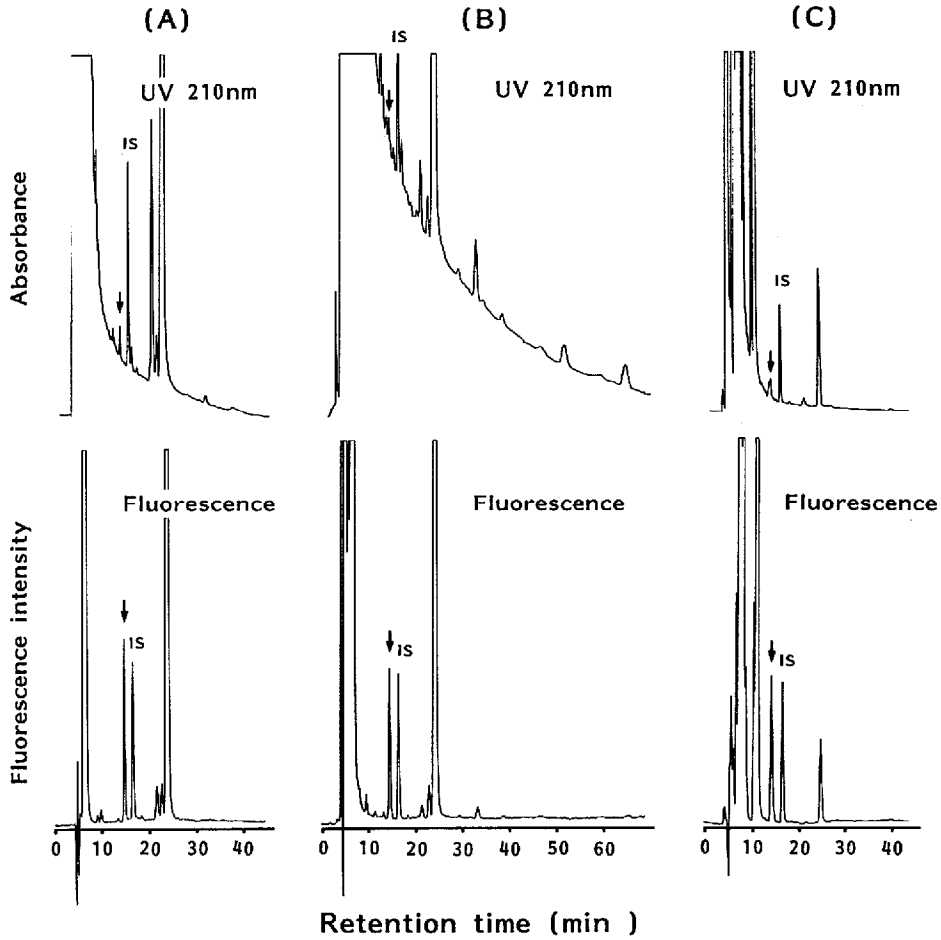

Fig. 5. Comparison of chromatograms for (A) the multivitamin tablet, (B) capsule and (C) frozen infusion obtained by the UV (210 nm) method (top) and the present fluorescence method (bottom). HPLC and post-column reaction conditions as in the text.

TABLE II

RECOVERIES OF BIOTIN FROM COMMERCIAL PHARMACEUTICAL PREPARATIONS

Preparation[a]	Biotin added (μg)	Biotin found[b] (μg)	Recovery (%)	Relative standard deviation (%)
Tablet	200	199.4 ± 5.3	99.7	2.63
Capsule	200	195.6 ± 4.4	97.8	2.18
Frozen infusion	200	194.2 ± 6.1	97.1	3.04

[a] See Table I.

[b] Each preparation was analysed five times; results are means ± S.D.

biotin, which are generally insensitive to UV at longer wavelengths, is seriously affected by various UV-absorbing substances, as the biotin peak appears in the tailing part of the large peaks of rapidly eluting compounds. This effect reduces the reliability of the determination. In contrast, the present method is less subject to interference from contaminants, because it is specific to the amide bonds. The chromatograms obtained by the present fluorescence method are simpler and give higher biotin peaks than those obtained using absorption at 210 nm. In addition, the peaks originating from the rapidly moving components are small enough that their tailing effect is negligible. Moreover, the present method shows only small fluctuations of the baseline and the chromatography is completed within 30 min.

The proposed method is expected to be useful for the sensitive and specific determination of biotin in various pharmaceutical products.

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