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Fluorescence detection of biotin using post-column derivatization with OPA in high performance liquid chromatography

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Abstract

A sensitive and selective high-performance liquid chromatographic method with post-column derivatization, using *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid (3-MPA), is described for the determination of biotin in pharmaceutical preparations. The use of 3-MPA gives intense fluorescent derivative and improves the stability of biotin fluorophore towards oxidation to the picomole level. The fluorophore was detected at 453 nm (excitation at 342 nm). The calibration graph was linear for 20–200 ng per injection. The detection limit of biotin under these conditions was about 10 ng per injection. The RSDs were 1.9–3.4%. This method could be applied to pharmaceutical preparations without interference of other compounds.

Keywords: Biotin; *o*-phthalaldehyde (OPA); Post-column derivatization; HPLC; Pharmaceutical preparations; Fluorimetric detection

1. Introduction

Biotin(*cis*-hexahydro-2-oxo-1-*H*-thieno-3,4-imindazoline-4-valeric acid; vitamin H) is a water soluble vitamin that is a coenzyme for carboxy-translocation reactions in enzymes, such as pyruvate carboxylase, acetyl-CoA carboxylase and methylcarboxyl CoA carboxylase and also plays a role in metabolism [1].

The current, widely accepted procedures for the determination of biotin in biological materials and in pharmaceutical preparations employ microbiological assays [2–4], which provide excellent sensitivity. However these methods are laborious and require a long incubation time.

The chemical methods including spectrophotometric methods based on dye-protein complexes [5], reaction with 4-dimethylaminocinnamaldehyde [6,7], and oxidation with potassium iodate [8] have been reported. However, these methods are neither sensitive nor specific as the microbiological methods. On the other hand, recently several high-performance liquid chromatographic (HPLC) procedures for the analysis of biotin have been developed [9–11]. At present the best HPLC methods include electrochemical detection [12]

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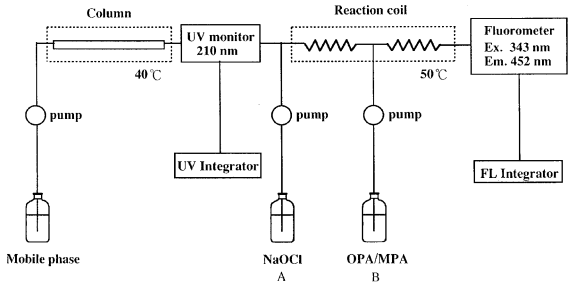

Fig. 1. Schematic diagram of the system for HPLC and fluorescence detection of biotin.

and fluorescent reaction with derivatization using such as 4-bromomethyl-7-methoxycumarin [13], anthryldiazomethane [14,15] and 1-pyrenyldiazomethane [16]. Although these methods are highly sensitive and specific, they are susceptible to interference.

The purpose of this study was to develop a sensitive and selective method to quantify biotin. The analyte after elution through the HPLC system was oxidized by hypochlorite solution, then formed the highly fluorescent derivative, using OPA and 3-MPA. In this report, we demonstrate the applicability to quantify biotin in pharmaceutical preparations.

2. Experimental

2.1. Chemicals

d-Biotin was supplied by Sigma (St. Louis, MO) and OPA, 3-MPA and sodium hypochlorite solution (Antiformin) and sodium dodecyl sulfate (SDS) were of analytical-reagent grade from Nacalai Tesque (Kyoto, Japan). Perchloric acid was purchased from Merk (Darmstadt, Germany). All other compounds were of reagent grade or better. Deionized, distilled water was used throughout the experiment.

2.2. Moble phase and derivatization reagents

The moble phase for HPLC was aqueous 20 mM SDS (pH 3.5 with perchloric acid)-acetnitrile (19:1, v/v). The solution was filtered through a 0.45 μm microfilter (Fuji, Tokyo, Japan) and degassed prior to use. The 0.03% hypochlorite reagent was prepared by adding 3 ml of Antiformin diluting with 0.2 M borate buffer (pH 12.5) to 1000 ml. The OPA reagent was prepared by dissolving 800 mg OPA in 20 ml ethanol, followed by addition of 1.5 ml 3-MPA diluting with 0.2 M borate buffer (pH 10.5) to 500 ml. 0.2 M Borate buffer solution was adjusted to pH 10.5 with 4 M sodium hydroxide solution. The reagent solutions were thoroughly degassed before use.

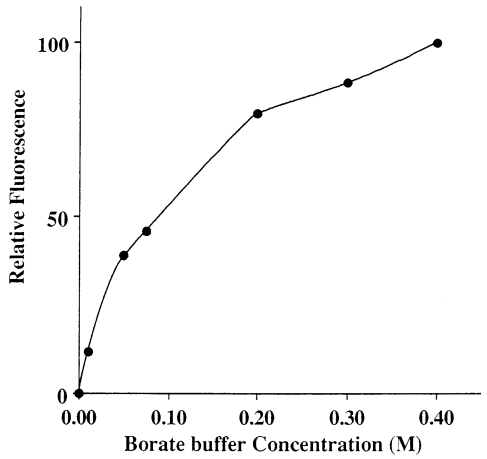

Fig. 2. Influence of borate buffer concentration on the fluorescence with biotin. HPLC and the post column reaction conditions as in the text.

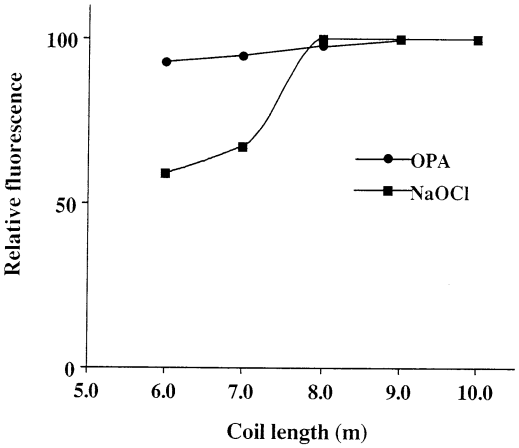

Fig. 3. Influence of length of the oxidation and reaction coil on the fluorescence with biotin. HPLC and the post column reaction conditions as in the text.

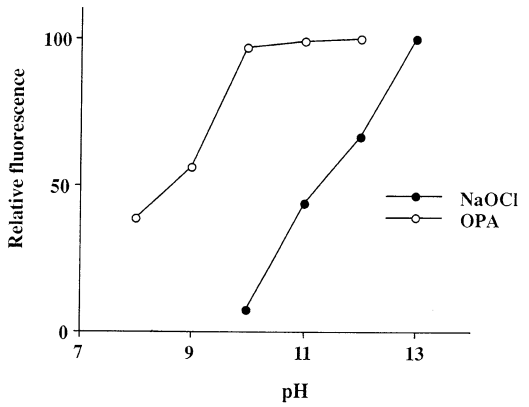

Fig. 4. Influence of pH in the oxidation and reaction solution on the fluorescence with biotin. HPLC and the post column reaction conditions as in the text.

Although the prepared OPA reagent is usually stable for more than 24 h, it is routinely used within 24 h in the author's laboratory to cautiously ensure that all chemicals used were of highest quality.

2.3. HPLC apparatus

Chromatographic separation was carried out on a Tomsorb C18 column (Tomsic, Tokyo, Japan, 5 μm, 4.6 mm i.d. × 250 mm) maintained at 40°C. The mobile phase was delivered using a Jasco Model 880-PU solvent pump (Japan Spectroscopic, Tokyo, Japan), at a flow rate of 0.5 ml min^{-1}. Samples were injected into the mobile phase stream via a Rheodyne injection Model 7125 (Cotati, CA, USA) valve with 20 μl loop. The column eluate first passed through a JASCO Model 875-UV detector set at 210 nm, and then it was introduced into the two step reaction coil. All the flow lines after the column were constructed with three way tees. From a reservoir (A), the hypochlorite reagent was delivered at a rate of 0.3 ml min^{-1} into the flow stream from the HPLC column. The two streams were mixed in a stainless-steel tube (10 × 0.5 mm i.d.), which was immersed in a thermoregulated water-bath maintained at 50°C. After oxidation, the OPA reagent was delivered from a reservoir (B) to the reaction stream at a flow rate of 0.3 ml min^{-1}. Then the mixture was passed through a stainless-steel tube (10 × 0.5mm i.d.) immersed in the same water-bath. The fluorescence intensity was measured at 453 nm with excitation at 342 nm in a Simadzu Model RT-10A spectrofluorimeter (Kyoto, Japan) equipped with a 10 μl flow cell. The chromatogram was recorded and integrated on a Shimadzu C-R 6A Chromatopac. Throughout this work, post-column derivatization experiments were carried out using the instrument arrangement shown in Fig. 1.

2.4. Analysis of multivitamin tablets and capsules

Twenty tablets and capsules containing biotin were weighed and finely powdered. An accurately weighed powder equivalent to about 200 μg of biotin was transferred into 50 ml flask and 30 ml water was added. The sample solution was ultrasonicated for 10 min. After being adjusted with water to 50 ml, it was centrifuged at 2000 × *g* for 10 min. The supernatant obtained was filtered through a 0.45 μm membrane filter and a 20 μl portion of the filtrate was injected into the system in Fig. 1.

3. Result and discussion

3.1. Effect of hypochlorite, 3-MPA and OPA concentration

In order to optimize the post-column reaction condition, an experiment was conducted by varying the concentrations of hypochlorite. The fluorescence intensity increased, corresponding to the amount of hypochlorite and reached a plateau at 0.02%.

The similar tendency of the effect of hypochlorite on the production of fluorescence was observed in the concentration of 3-MPA and OPA. Therefore, the concentrations of OPA and 3-MPA were adjusted to 10 mM and 0.6% (v/v), respectively.

P. Bohlen [17] has claimed that sodium hypochlorite, under alkaline conditions, converts the imino compounds to substances which generate fluorescence upon reaction with OPA. The

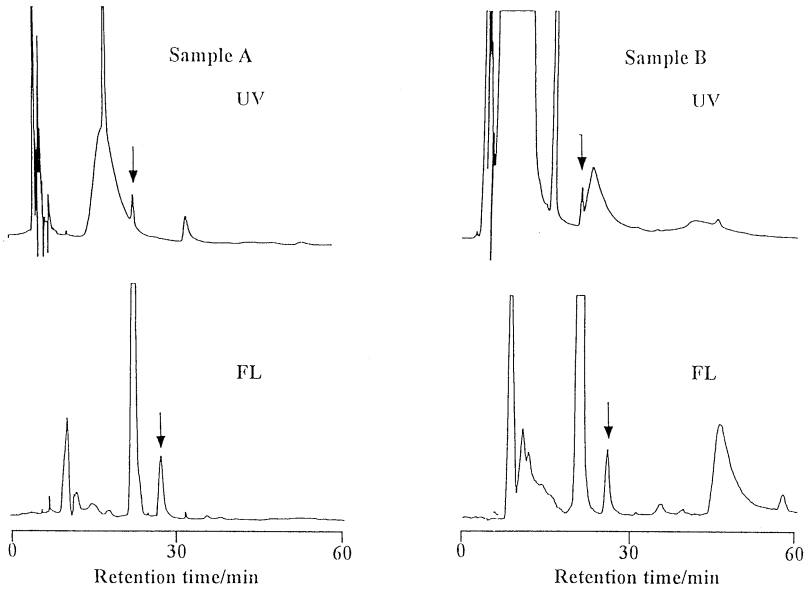

Fig. 5. Comparison of chromatograms obtained by the UV (210 nm) method (top) and the present method (bottom). HPLC and the post column reaction conditions as in the text. Sample 80 μg ml^{-1} of biotin, 20 μl injection.

nature of the conversion products of biotin has not been elucidated; it is reasonable to assume that biotin, including the imino ring, is derivatized with OPA-3-MPA through oxidation in a similar reaction process of imino acids as A. Fiorino reported. [18].

In addition, we found that borate buffer concentration was also affected to this reaction. An increase in fluorescence is observed with the increasing concentration of borate (Fig. 2). However, above 0.3 M borate buffer, flow blockage was observed in the reaction coil. Therefore, 0.2 M borate buffer was chosen for the oxidation solution and the reagent solution.

3.2. Influence of the reaction temperature and the coil length

The fluorescence intensity of the biotin signal was also dependent on the reaction temperature and the length of reaction coil. Efforts were made to investigate the effect of the reaction temperature. Fluorescence intensity increasesd with the reaction temperature. At room temperature the reaction of biotin with hypochlorite was very slow, whereas appreciable fluorescence could be generated at 50–60°C. Above 60°C, a large bubble formation in the flow line, which caused noises on the chromatogram, was occurred, so that the reaction temperature was then set at 50°C.

In order to study the effect of length of the oxidation and the reaction coil, an investigation in the coil length ranged from 6 to 10 m was carried out. From this study, an optimal length of the oxidation coil ranged between 8 and 10 m and that of the reaction coil ranged between 9 and 10 m were obtained (Fig. 3). The use of a reaction coil longer than 10 m led to peak broadening, but the peak areas remained constant. Therefore, 10 m coils of oxidation and reaction were employed in this system; the oxidation and the reaction times were about 60 s.

3.3. Effect of pH

Study on the effect of pH on the oxidation of biotin was carried out. As shown in Fig. 4, an increase in fluorescence is observed with higher

Table 1
Recoveries of biotin from pharmacetical preparations

Lot	Preparation	Added	Recovery (% ± SD)[a]	Ingredients
1	Tablet	50 μg T^{-1}	100.1 ± 1.70	Riboflavin Octotiamine
		100	99.8 ± 1.41	Pyridoxin hydrochloide Cyanocobalamin
2	Capsule	50 μg $Cap.^{-1}$	99.6 ± 2.55	Fursultiamine hydrochloride Hydroloxobalmin lactate
		100	100.0 ± 1.75	Pyridoxal phosphate Riboflavin
3	Granule	50 μg $Pack.^{-1}$	99.2 ± 1.75	Riboflavin phosphate Pyridoxal hydrochloride
		100	99.7 ± 1.52	Bisbentiamine Coix seed

[a] Mean of three determinations ± SD.

pH. This phenomenon may be explained due to pH dependency on the oxidation process. In addition, the effect of pH on the reaction solution was also investigated. Although fluorescence is stable above pH 10, the reagent solution itself tends to possess fluorescence higher than pH 11. Consequently, the pH of the oxidation and the reagent solution were adjusted to 12.5 and 10.5, respectively.

3.4. Quantitative properties

The chromatogram obtained by this method was simpler and gave a higher peak of biotin than that obtained by using absorption at 210 nm (Fig. 5). Absorption at 210 nm tended to be seriously affected by various UV-absorbing substances when assaying biotin, so that the biotin peak was easily interfered with peaks of rapidly eluting compounds. Compared to the UV detection method, the present method is less subject to interference from other compounds, because of specificity to the secondary amines.

Calibration for biotin showed excellent linearity in the range from 20–200 ng per injection. The reproducibility of this procedure in terms of retention time and peak area were also sufficient, the RSD being 1.3% ($n = 5$) for 100 ng per injection of standard samples of biotin. The detection limit of biotin under these conditions was about 10 ng per injection (signals-to-noise ratio, $S/N = 3$).

The present standard procedure was applied to the determination of biotin spiked in pharmaceutical preparations. Biotin was recovered completely and could be determined with good precision as shown in Table 1. The recovery of biotin was 96.4–101.1% with RSD of 1.9–3.4% ($n = 3$).

4. Conclusion

The proposed method appears to be suitable for the determination of biotin in various pharmaceutical products with good precision and reproducibility.

Moreover, this method shows only small fluctuations of base line and HPLC separation followed by post-column fluorescence derivatization provides a simple and time-saving method, with a chromatographic running time of less than 30 min. Another advantage of this procedure is that there is no need for pre-column purification or removal of excess reagent prior to HPLC analysis.

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