TLC Determination of Biotin in a Lyophilized Multivitamin Preparation

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Abstract □ A TLC method for the determination of biotin was developed. The method is selective and sensitive and permits the determination of biotin in the presence of all water-soluble vitamins as well as glycine, edetate sodium, and methylparaben. The main steps are: dissolution of the lyophilized multivitamin preparation in water, addition of the internal standard, application on a thin-layer plate, separation from interfering components, visualization by spraying with 4-dimethylaminocinnamaldehyde, and *in situ* determination by reflectance measurements. Spraying the plate with paraffin after the coloring procedure increases the sensitivity of the method. The coefficient of variation is 2.3% with 0.15 μg of biotin/spot. Quantities as low as 10 ng/spot can be determined.

Keyphrases □ Biotin—TLC analysis in lyophilized multivitamin preparation □ TLC—analysis, biotin in lyophilized multivitamin preparation □ Vitamins—biotin, TLC analysis in lyophilized multivitamin preparation

Microbiological methods generally are used to determine biotin in small amounts (1–5), but bioassays also have been used (6). The chemical and physicochemical methods are spectrophotometric assays based on the binding of a dye by avidin (7), oxidation with potassium iodate (8), or reaction with 4-dimethylaminocinnamaldehyde (9, 10). GLC after silylation with bis(trimethylsilyl)acetamide (11–14), polarography (15, 16), and isotope dilution (9, 17, 18) also are used.

None of the chemical and physicochemical methods was applicable to a lyophilized multivitamin preparation because of a lack of sensitivity or interference from other components. However, the use of 4-dimethylaminocinnamaldehyde as a spray reagent for biotin after separation by TLC or paper chromatography has been proposed (9, 19). This paper reports the conditions for *in situ* quantitation of biotin in a complex mixture after TLC separation and coloration with 4-dimethylaminocinnamaldehyde.

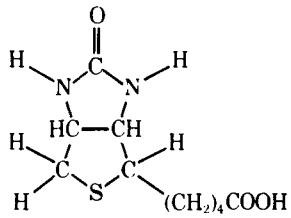
EXPERIMENTAL

Apparatus—A dual-wavelength chromatogram scanner[1] was used in the *in situ* reflection measurements. Sample and reference wavelengths were 530 and 400 nm, respectively. The scanning was performed in the zig-zag mode at 10 mm/min with a 1.25 × 1.25-mm slit. A two-channel[2] recorder, range 10–20 mv, also was used.

Silica gel 60 precoated plates[3] (20 × 20 cm) were developed in a sealed chromatographic chamber, lined with filter paper to obtain a saturated atmosphere. A polytef-tipped 10-μl syringe[4] and a home-made precision device were used to obtain reproducible spotting of the sample and standard solutions on the plates.

The coloring reagent and paraffin solution were applied with all-glass sprayers.

Substances and Reagents—The biotin[5] (I) standard solution contained 0.12 mg/ml of water, and the 2-imidazolidone[5] (II) internal standard solution contained 0.15 mg/ml of water. The eluent was chloroform[3]–methanol[3]–formic acid[3] (70:40:2, v/v/v).



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The reagent spray solution contained a mixture of 0.1% (w/v) 4-dimethylaminocinnamaldehyde[6] and 1% (v/v) sulfuric acid in ethanol. The paraffin spray solution contained 10% (v/v) liquid paraffin USP in chloroform[3].

Preparation of Sample and Standard Solutions—To separate 10-ml volumetric flasks were transferred 1.00, 2.00, 3.00, and 4.00 ml of biotin standard solution. Then 0.50 ml of internal standard solution was added to each flask, and the contents were diluted to the mark with water.

To the sample bottle containing the lyophilized multivitamin powder, 0.50 ml of internal standard solution and 9.50 ml of water were added. The bottle was shaken to dissolve the powder.

TLC—Aliquots of 5 μl of sample and standard solutions were applied to the TLC plate, 2 cm from the lower edge, and the spots were dried carefully. The chromatogram was developed in a saturated atmosphere (saturation overnight), and the solvent front migrated 15 cm above the application line.

After removal of the plate from the chamber, it was dried in a stream of cold air for about 5 min and then sprayed homogeneously with reagent solution until the spots appeared distinct red to red-violet. After the plate was again blown with a stream of cold air for about 5 min, it was sprayed homogeneously with paraffin solution until there was no visual increase in color intensity. ("Wet" layers were avoided.) Chloroform was removed from the plate with a stream of cold air. The chromatogram was scanned

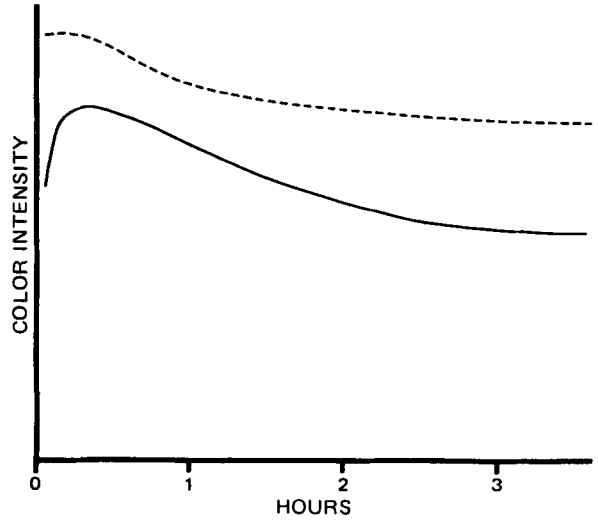

Figure 1—*Color development after spraying with 4-dimethylaminocinnamaldehyde. The color intensity was measured as the peak area at 530 nm. Key: —, biotin; and - - -, 2-imidazolidone.*

[1] Model CS-900, equipped with a background correction unit (BCU-1), Shimadzu, Tokyo, Japan.
[2] Servogor 320, Goerz Electro G.m.b.H., Vienna, Austria.
[3] E. Merck, Darmstadt, West Germany.
[4] Unimetrics Corp., Anaheim, Calif.
[5] Fluka AG., Buchs, Switzerland.
[6] Sigma Chemical Co., St. Louis, Mo.

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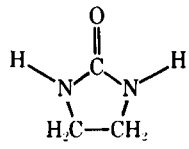

II

in the direction of the development 4 hr after spraying with paraffin solution.

A standard graph was plotted with the peak area ratio of biotin to the internal standard as the ordinate against the total amount of biotin in the standard solutions. By using the peak area ratio of the sample, the amount of biotin in the sample bottle was obtained directly from this standard graph.

RESULTS AND DISCUSSION

Color Reaction—Biotin (I) and other cyclic ureido compounds react with 4-dimethylaminocinnamaldehyde in nonaqueous media (or on dry chromatograms) to form Schiff bases (9). This reaction is stereospecific and involves the less sterically hindered N_1 of the ureido portion of biotin. The monocyclic ureide, 2-imidazolidone (II), used as an internal standard, is less hindered sterically and gives a more intense color than biotin.

The composition of the lyophilized multivitamin preparation is given in Table I. A colorimetric determination of biotin with 4-dimethylaminocinnamaldehyde added directly to the sample mixture, as described by Shimada *et al.* (9), failed because of interference from the other components. However, 4-dimethylaminocinnamaldehyde appeared to be an excellent reagent for visualization of biotin and 2-imidazolidone after separation from interfering components on silica gel thin-layer plates, where they were seen as red and red-violet spots, respectively. Attempts to perform the reaction before chromatography and subsequent separation of the products failed because of color instability and nonhomogeneous spots.

Figure 1 shows the color development of TLC spots after spraying with 4-dimethylaminocinnamaldehyde and paraffin. Maximum color intensity was reached after 20–25 min for biotin and after 5–10 min for 2-imidazolidone. The relation between color intensities of equimolar amounts of 2-imidazolidone and biotin was about 3:2. The color faded with time but seemed to stabilize after about 3–4 hr. In fact, spots scanned after more than 24 hr showed about the same intensity as after 3–4 hr. Spraying with paraffin increased the color intensity measured at 530 nm about three times and stabilized (prevented discoloration of) the background.

Table I—Composition of the Lyophilized Multivitamin Preparation

Compound	Milligrams per Flask
Thiamine mononitrate	1.24
Riboflavin phosphate (sodium)	2.47
Niacinamide	10
Pyridoxine hydrochloride	2.43
Sodium pantothenate	11
Sodium ascorbate	34
Biotin	0.3
Folic acid	0.2
Cyanocobalamin	0.002
Glycine	100
Edetate sodium	0.5
Methylparaben	0.5

Table II—Quantitative Results from Repeated Determinations of a Lyophilized Vitamin Sample

Number[a]	Milligrams of Biotin per Flask[b]
1	0.356
2	0.339
3	0.345
4	0.350
5	0.350
6	0.335
7	0.346
8	0.354
9	0.340
10	0.332
Mean	0.345
CV, %	2.3
Recovery, %[c]	101

[a] Different thin-layer plates. [b] Mean of two sample spots per plate. [c] Obtained by addition of biotin to a standard vitamin mixture (see Table I).

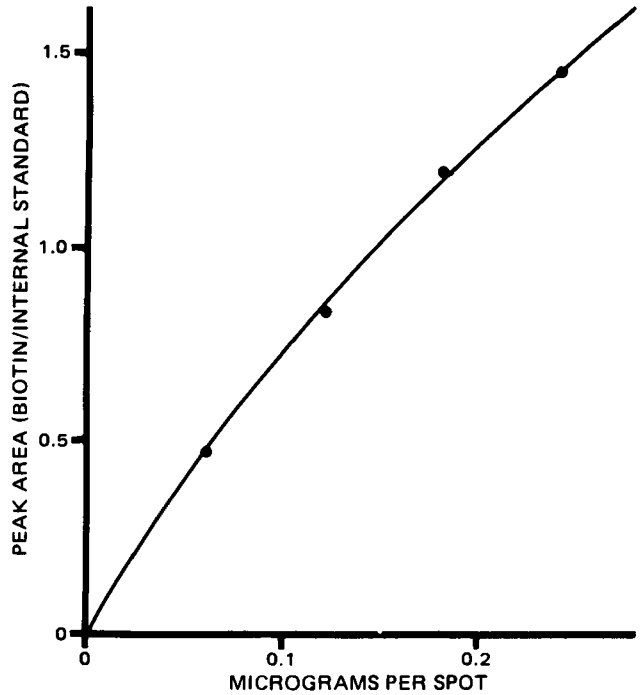

Figure 2—*Standard graph of biotin after spraying with 4-dimethylaminocinnamaldehyde and liquid paraffin;* λ_{sample} = *530 nm and* $\lambda_{reference}$ = *400 nm. Scanning was in the zig-zag mode.*

TLC Procedure—The atmosphere in the chromatographic chamber must be carefully saturated. It was convenient to saturate overnight. Incomplete saturation caused tailing (or at least asymmetrical) spots and incomplete separation between biotin and the internal standard. Furthermore, the composition of the eluent was critical for the separation.

The relative R_f (biotin to internal standard) was about 1.2, with the R_f values of biotin and internal standard being approximately 0.55 and 0.45, respectively. Separation was somewhat reduced in the presence of the sample components because of a slight change in shape of the biotin spot. The addition of glycine (Table I) to the standard solutions did not affect the spot shape remarkably. Niacinamide migrated between biotin and the internal standard, and sodium pantothenate migrated closely above the biotin spot in the chromatogram. However, they did not interfere in the determinations since they did not react with 4-dimethylaminocinnamaldehyde.

***In Situ* Quantitation**—Quantitation was performed by scanning in the reflectance mode. Because of the differences in spot shape already mentioned, zig-zag scanning yielded the most accurate results (20). Measurements in the transmission mode increased the sensitivity by a factor of about two; but due to higher background disturbances, the reflectance mode was chosen.

The standard graph (Fig. 2) is slightly curved. The reason for this nonlinearity was discussed previously (20–22).

The recovery of the method was determined by addition of a known amount of biotin to a standard vitamin mixture. The precision was calculated from repeated determinations of a sample solution. The results are presented in Table II. Biotin was recovered completely and could be determined with good precision without interference from the other components.

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Determination of Ketoprofen by Direct Injection of Deproteinized Body Fluids into a High-Pressure Liquid Chromatographic System

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Received April 24, 1978, from *"A. Menarini" Pharmaceuticals, Via Sette Santi 1, 50131 Florence, Italy.* Accepted for publication August 11, 1978.

Abstract □ A rapid, sensitive, and specific determination of ketoprofen in human and animal deproteinized body fluids by reversed-phase high-pressure liquid chromatography is presented. The acid is detectable in amounts as low as 0.1 μg/ml. Limits of error are in the range of ±5.1% of the sample mean.

Keyphrases □ Ketoprofen—high-pressure liquid chromatographic analysis in various body fluids □ High-pressure liquid chromatography—analysis, ketoprofen in various body fluids □ Anti-inflammatory agents—ketoprofen, high-pressure liquid chromatographic analysis in various body fluids

A TLC procedure (1) for the determination of ketoprofen, 2-(3-benzoylphenyl)propionic acid, in body fluids overcame most limitations of other procedures (2). The search for new methods to shorten the time required to perform analyses and to improve sensitivity led to the present high-pressure liquid chromatographic (HPLC) procedure.

EXPERIMENTAL

Apparatus and Operating Conditions—A high-pressure liquid chromatograph[1] was equipped with two independent pumps, a reversed-phase column[2] (0.25 m × 4 mm), a variable wavelength spectrophotometer[3] to monitor the column effluent, and a liquid chromatograph terminal[4] to program chromatography conditions and record and integrate peak areas. Operating conditions were adjusted to give a mobile phase flow rate of 0.8 ml/min (operating resulting pressure of 32–38 atm). The column oven temperature was set at 40°. The detector wavelength was 255 nm (for quantitative evaluation) or 205 nm (for qualitative evaluation of metabolites).

Table I—Recovery of Ketoprofen Added to Human Plasma as Compared to Ketoprofen Dissolved in Phosphate Buffer (255 nm)

Ketoprofen Added, μg/ml	Relative Area		Recovery, %
	Buffer	Plasma	
1	16.760	13.946	83.9
	16.500	13.685	
	17.040	13.750	
	15.500	13.800	
	16.450	13.795	
2	33.000	31.822	90.4
	34.590	29.370	
	35.670	29.582	
	32.730	32.103	
	33.998	30.719	
4	68.700	64.428	90.3
	72.400	63.862	
	71.500	61.596	
	71.100	65.844	
	70.800	63.933	
8	140.320	127.430	90.7
	139.570	128.010	
	139.870	127.100	
	142.010	126.750	
	140.443	127.323	

Reagents—Methanol[5] for HPLC was used as received. Double-distilled water was filtered through a 0.2-μm pore size membrane filter[6]. The mobile phase was double-distilled water–methanol (85:15).

Preparation of Standards—Ketoprofen was dissolved in 96% ethanol or 0.15 *M* phosphate buffer at pH 8. Aliquots of ethanolic solutions equivalent to 1, 2, 4, and 8 μg were placed in glass-stoppered centrifuge tubes and evaporated to dryness under a gentle nitrogen stream. Then 1 ml of untreated body fluid was added to each tube and mixed well.

[1] Hewlett-Packard 1084A.
[2] Hewlett-Packard RP8-79918A.
[3] Hewlett-Packard 1030B.
[4] Hewlett-Packard 79850A.
[5] Merck.
[6] Sartorius type SM 11307.

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