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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIOTIN AND ANALOGUES

JANE L. CHASTAIN*, DELORES M. BOWERS-KOMRO and DONALD B. McCORMICK
Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322 (U.S.A.)
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SUMMARY

Biotin, analogues, and chemical intermediates were separated by high-performance liquid chromatography (HPLC) using reversed-phase and anion-exchange chromatographic conditions. Reversed-phase separations provided a wide range of retention times and resolution of nearly all the biotin compounds from mixtures of the analogues. Anion-exchange separations gave generally shorter retention times as compared to reversed-phase separations and greater resolution between biotin *l*- and *d*-sulfoxide. However, fewer analogues were resolved from mixtures of the compounds with anion-exchange HPLC.

INTRODUCTION

Biotin and analogues have been isolated and characterized by several investigators[1] using gravity flow anion-exchange, thin-layer, and paper chromatography[2,3]. Desbene *et al.*[4] have separated biotin and analogues by high-performance liquid chromatography (HPLC) on a bonded phase C_{18} column by derivatizing the compounds to ω,4-dibromoacetophenone esters (UV detection) and 4-bromomethylmethoxy-coumarin derivatives (fluorometric detection). HPLC detection of biotin in pharmaceutics has been reported by Roder *et al.*[5]. In this paper, the separation of underivatized biotin, analogues, and chemical intermediates by reversed-phase and anion-exchange HPLC is investigated.

EXPERIMENTAL

Instrumentation

Separations were made using a Waters Assoc. (Milford, MA, U.S.A.) HPLC system consisting of the following: 6000A and M45 solvent delivery system with 720 system controller, 450 variable-wavelength detector, U6K injector, and M730 data module. HPLC columns used on the above system were: (1) 10-μm μBondapak C_{18} column (250 × 4.6 mm I.D., Waters Assoc.) with RP-C_{18} guard column (30 × 4.6 mm I.D., Brownlee Labs, Santa Clara, CA, U.S.A.), (2) Aquapore AX-300 column (250 × 4.6 mm I.D.) with AX-300 guard column (30 × 4.6 mm I.D., Brownlee

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Labs.), and (3) Partisil SAX column (250 × 4.6 mm I.D., Chromanetics, Gaithersburg, MD, U.S.A.).

Elution patterns were monitored at 220 nm to detect the carbonyl function of the biotin molecule. Solvents were selected to give minimal background interference at this wavelength. Sensitivity was kept at 0.04 a.u.f.s. unless otherwise specified.

Materials

Biotin and analogues (both chemically and catabolically derived) were obtained through previous work on the metabolism of the vitamin[1]. Thiophanium bromide, thiophanium camphor *d*-sulfonate, imidazolidone *cis*-dicarboxylic acid, and thiolactone were obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Polar biotin compounds were dissolved in 0.004 *M* sodium hydroxide, whereas less polar compounds were dissolved in water–acetonitrile (50:50, v/v).

Sulfoxides of tetranorbiotin and bisnorbiotin were formed by reacting 100 μl of 1.0 mg/ml tetranor- or bisnorbiotin solution with an equal volume of hydrogen peroxide solution (0.34 ml 30% hydrogen peroxide in 10 ml 0.1 *M* hydrochloric acid) at room temperature. To detect sulfoxide formation, 20-μl aliquots of reaction mixture were injected onto the μBondapak C_{18} column at time zero, 60 min and overnight. Sulfones of tetranorbiotin and bisnorbiotin were prepared and detected in the same manner as for sulfoxides except that a five times greater amount of hydrogen peroxide was used.

Chromatographic conditions

The solvent system for elution on μBondapak C_{18} column consisted of aqueous 0.05% trifluoroacetic acid (TFA) (adjusted to pH 2.5 with potassium hydroxide) and 0.05% TFA–acetonitrile (50:50, v/v). Solvents were filtered through a 0.45-μm membrane filter (Rainin Instruments, Woburn, MA, U.S.A.) under vacuum prior to use. Separations were made at a flow-rate of 1.0 ml/min using a 35-min linear gradient beginning with 0.05% TFA and ending with a final 0.05% TFA–acetonitrile ratio of 70:30 (v/v). The 70:30 solvent ratio was held for 5 min. A 5-min column equilibration period with the initial solvent was needed between runs to achieve good reproducibility.

The solvent system for anion-exchange separations on Aquapore AX-300 and Partisil SAX columns was 0.05 *M* Tris–HCl at pH 4.5 and 6.5. Separations were made at a flow-rate of 1.0 ml/min with a 30-min pH gradient with an initial pH of 4.5 and final pH of 6.5. A 10-min wash between runs with 0.05 *M* Tris, pH 4.5, re-equilibrated the column.

RESULTS

Retention times (in min) of biotin and analogues are given in Table I. All compounds were injected singly and in mixtures to determine the degree of resolution achieved.

Reversed-phase separations

Retention times on μBondapak C_{18} column ranged from 4.8 to 37.0 min. The number of methylene carbons present in the side chain of biotin has a pronounced

TABLE I

RETENTION TIMES OF BIOTIN AND ANALOGUES

Compound	*pK$_a$**	*Retention time (min)*		
		*μBondapak C_{18}***	*Aquapore AX-300****	*Partisil SAX****
Biotin	6.22	26.0	17.7	12.6
Homobiotin	6.29	32.2	18.4	13.3
Bisnorbiotin	5.86	15.5	16.7	11.3
Tetranobiotin	4.85	6.1	15.3	8.3
α-Dehydrobiotin		25.2	22.3	12.7
Biotinol		27.2	3.9	5.2
Biocytin		19.0	3.7	6.0
Biotin methyl ester		37.0	3.8	6.1
Biotin *l*-sulfoxide	6.05	13.3	13.5	14.8
Biotin *d*-sulfoxide	6.04	14.1	12.1	9.3
Biotin sulfone	6.10	4.8	14.8	10.5
Dethiobiotin	6.35	30.2	14.0	12.4
2′-Thiobiotin		25.8	23.7	12.5
2′-Iminobiotin	12.0	4.5–5.0	4.3	6.7

* pK_a values taken from Sigel *et al.*[6].

** Separations were made with a 35-min linear gradient beginning with aqueous 0.05% TFA (adjusted to pH 2.5 with potassium hydroxide) and ending with a 0.05% TFA–acetonitrile ratio of 70:30. The 70:30 ratio was held for 5 min. Flow-rate was 1.0 ml/min.

*** Separations were made using a 30-min linear pH gradient beginning with 0.05 *M* Tris–HCl at pH 4.5 and ending with a final pH of 6.5. Flow-rate was 1.0 ml/min.

effect on elution time from the column. In general, as the number of methyl groups in the side chain increases, retention time increases. The relationship is linear with a correlation coefficient of 0.997. Tetranorbiotin, with no side-chain methylene carbons, elutes at 6.1 min followed by bisnorbiotin (15.5 min), biotin (26.0 min), and homobiotin (32.2 min), each with 2, 4 and 5 side-chain methylene carbons respectively.

The presence of a double bond in the side chain of biotin, *viz.* α-dehydrobiotin, has little effect on retention time. α-Dehydrobiotin elutes closely with biotin (25.2 *vs.* 26.0 min) and is unresolved from biotin in a mixture of these two compounds.

Biotin methyl ester is less polar than biotin and is well retained on the C_{18} column (37.0 min). However, the lysine moiety present on the side chain of biocytin increases the polarity causing it to elute more rapidly (19.0 min). Altering the acid functionality of biotin to an alcohol (biotinol) slightly lengthens retention on the column from 26.0 min (biotin) to 27.2 min. Although biotin and biotinol elute closely together, both compounds are resolved when injected as a mixture onto the C_{18} column.

Polarity of the biotin molecule increases when the sulfur moiety of the thiophane ring is oxidized to sulfoxide and sulfone. Retention times for biotin sulfone, biotin *l*-sulfoxide, and biotin *d*-sulfoxide (4.8, 13.3, and 14.1 min, respectively) are decreased compared to unoxidized biotin. Removal of the sulfur from the thiophane ring system to form dethiobiotin causes a decrease in polarity of the biotin molecule and lengthens retention on the column to 30.2 min.

Replacing the 2′-oxygen in the biotin ureido ring system with sulfur to form 2′-thiobiotin does not significantly alter retention on the column from that of biotin (25.8 *vs.* 26.0 min). However, introduction of an imino group in the 2′-position (2′-iminobiotin) markedly shortens retention on the column (4.5–5.0 *vs.* 26.0 min). The guanidino-like ring structure of 2′-iminobiotin has a pK_a near 12 and is protonated under chromatographic conditions used for the C_{18} column, causing the compound to elute more rapidly than biotin.

Anion-exchange separations

Retention times on the Aquapore AX-300 column (weak anion exchanger) ranged from 3.7 to 23.7 min. The linear relationship between number of side-chain methylene carbons and retention time existed with the Aquapore column as with C_{18} except that the difference between retention times for the four homologues (tetranorbiotin, bisnorbiotin, biotin, and homobiotin) was not as great. Also, tetranorbiotin falls below the line produced by the other three homologues; this is possibly a function of its relatively low pK_a.

α-Dehydrobiotin was well-resolved from biotin on the Aquapore AX-300 column (23.3 *vs.* 17.7 min); however, biotinol, biocytin, and biotin methyl ester eluted rapidly and could not be resolved when a mixture of the three compounds was injected.

Retention times of biotin *l*-sulfoxide, biotin *d*-sulfoxide, and biotin sulfone more closely followed differences in pK_a values; lower values generally lead to shorter retention times. Dethiobiotin eluted more rapidly on the Aquapore as compared to the C_{18} column (14.0 *vs.* 30.2 min).

Changing the 2′-position of biotin to 2′-thiobiotin altered the ionic nature of the molecule causing slower elution from the Aquapore column and, thus, greater differences in retention times between biotin and 2′-thiobiotin. 2′-Iminobiotin with its relatively high pK_a elutes rapidly as expected for this cationic species.

Retention times using the Partisil SAX column (strong anion exchanger) ranged from 5.2 to 14.8 min. Peaks eluted from the SAX column were sharper and more well-defined than on the Aquapore column; however, fewer analogues were resolved. Biotin, α-dehydrobiotin, dethiobiotin, and 2′-thiobiotin eluted together in a single peak as did biocytin, biotin methyl ester, and 2′-iminobiotin.

The relationship of number of side-chain methylene carbons *vs.* retention time was evident using the SAX column, with the range of retention times greater than with the Aquapore column but not as pronounced as with reversed-phase separations.

Biotin *d*-sulfoxide, biotin sulfone, and dethiobiotin elute from the SAX column according to differences in pK_a, the exception being biotin *l*-sulfoxide which elutes at 14.8 min. Differences in retention time between biotin *d*-sulfoxide and biotin *l*-sulfoxide (5.5 min) cannot be accounted for by the inconsequential difference in pK_a values for the two isomers: therefore, other factors affect elution of biotin *l*-sulfoxide from the SAX column.

Separation of tetranorbiotin and bisnorbiotin sulfoxides

A clear relationship exists between length of the biotin side chain and retention time with all the separation methods and was most evident with reversed-phase sep-

TABLE II

RETENTION TIMES OF BIOTIN CHEMICAL SYNTHESIS INTERMEDIATES

Compound	*Retention time (min)*	
	*35:65**	*25:75**
Thiolactone	5.8	4.6
Imidazolidone *cis*-dicarboxylic acid	4.0	3.5
Thiophanium bromide	7.3**	8.5
Thiophanium camphor *d*-sulfonate	7.3	8.5

* Ratio of aqueous 0.05% TFA–acetonitrile.
** Peak broadening of the thiophanium intermediates occurred with the 35:65 ratio of solvent.

arations. It was desirable to determine if forming sulfoxides of the homologues with different numbers of methyl groups in their side chains would affect their elution behavior. Sulfoxides and sulfones of tetranorbiotin and bisnorbiotin (see Experimental) were injected onto the C_{18} column. Evidence for sulfoxide formation was the disappearance or a decrease in area for known peaks of tetranorbiotin and bisnorbiotin and the emergence of new peaks proportional to the decrease in area of known analogues. Bisnorbiotin *d*-sulfoxide had a retention time of 6.4 min while bisnorbiotin sulfone as well as tetranorbiotin sulfoxide and sulfone eluted rapidly with the solvent front and were unable to be separately detected.

Separation of biotin chemical intermediates

Thiophanium bromide, thiophanium camphor *d*-sulfonate, thiolactone, and imidazolidone *cis*-dicarboxylic acid are intermediates in the commercial synthesis of biotin as developed by Hoffmann-LaRoche[7]. These compounds were separated on the C_{18} column using two isocratic solvent systems with differing ratios of 0.05% TFA–acetonitrile. Results are presented in Table II. The 35:65 TFA–acetonitrile ratio yields a greater difference in retention times of thiolactone and imidazolidone *cis*-dicarboxylic acid than the 25:75 ratio. However, peak broadening of the thiophanium intermediates occurred at this lower concentration of acetonitrile. Overall, a 25:75 solvent ratio gave better separations in terms of resolution and peak sharpness.

DISCUSSION

Reversed-phase chromatographic conditions provided a wider range of resolution than anion-exchange conditions and resolved nearly all the analogues analyzed in this study. Greatest differences in retention times were observed for biotin analogues with variations in the side chain and in the thiophane ring. The lower limit of detection for biotin on the C_{18} column is 10^{-6} g/ml at a sensitivity of 0.01 a.u.f.s. Detection limits of 10^{-8} g/ml for biotin on a bonded phase C_{18} column have been reported by Desbene *et al.*[4]; however, achievement of this sensitivity involved derivatizing biotin with ω,4-dibromoacetophenone to produce the UV-absorbing ester.

Anion-exchange conditions separated biotin and analogues with generally shorter retention ranges, but fewer analogues were resolved. Other solvent systems including aqueous isoprotic Tris and phosphate buffers (pH 4.5, 5.0, 5.5, and 6.5)

with and without gradients to increase ionic strength using 0.5 *M* sodium chloride were tried to increase resolution but with little success. Anion-exchange columns provide a greater degree of resolution for the *l*- and *d*-isomers of biotin sulfoxide than the C_{18} column. Possibly, greater resolution of the analogues could be achieved if flow-rates were decreased and the mixtures not overly complex.

In conclusion, biotin and analogues can be separated underivatized with HPLC using both reversed-phase and anion-exchange chromatographic conditions. Separation of more complex biotin mixtures would best be accomplished by reversed-phase HPLC, since the range of retention times is somewhat larger. Anion-exchange HPLC would be better utilized to separate simple mixtures in a relatively short amount of time and to separate sulfoxide isomers.

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