ELSEVIER

Lipoic acid (thioctic acid) analogs, tryptophan analogs, and urea do not interfere with the assay of biotin and biotin metabolites by high-performance liquid chromatography/avidin-binding assay

Janos Zempleni, Donald B. McCormick,* Shawna L. Stratton, and Donald M. Mock

*Dept. of Pediatrics, University of Arkansas for Medical Sciences and the Arkansas Children's Hospital Research Institute, Little Rock, AR; and *Dept. of Biochemistry, School of Medicine, Emory University, Atlanta, GA*

Lipoic acid, urea, and tryptophan show structural similarities to the vitamin biotin. If these compounds can successfully compete with biotin or a biotinylated protein for binding to avidin, their presence in serum and urine would cause artifacts in avidin-binding assays for biotin. We assessed the ability of lipoic acid and certain analogs, urea, and L*-tryptophan and tryptophan derivatives to interfere with the measurement of 16 biotin analogs by a high-performance liquid chromatography (HPLC)/avidin-binding assay. In this assay, compounds are separated by reversed-phase HPLC, followed by assay of each fraction based on binding to avidin-horseradish peroxidase. At physiologic concentrations, neither lipoic acid analogs (*d*-lipoate,* l*-lipoate,* d,l*-lipoamide, bisnorlipoate, β-hydroxybisnorlipoate, and tetranorlipoate) nor tryptophan derivatives exhibited detectable avidin-binding. Minor avidin-binding was seen for urea and* L*-tryptophan; binding ratios relative to biotin were* 1×10^{-9} *and* 3×10^{-6}*, respectively. Urea did not co-elute on HPLC with any of the 16 biotin analogs, but* L*-tryptophan did co-elute with bisnorbiotin methyl ketone. However, because the relative avidin affinity of* L*-tryptophan is five orders of magnitude smaller than that of bisnorbiotin methyl ketone, we conclude that none of the tested compounds are likely to interfere with the measurement of biotin or its metabolites by an HPLC/avidin-binding assay.* (J. Nutr. Biochem. 7:518–523, 1996.)

Keywords: lipoic acid; tryptophan; urea; biotin; avidin-binding assay; high-performance liquid chromatography

Introduction

For normal subjects, the mean concentrations of biotin and its metabolites in human plasma and serum are less than 1 nmol/L.[1] Hence, sensitive detection techniques such as avidin-binding assays and bioassays are required for accurate quantitation. Avidin-binding assays are generally performed in one of two ways: (1) isotope dilution of radiolabeled biotin[2–10] or (2) biotin binding to avidin (or streptavidin) labeled with one of several reporter molecules.[1,11,12] Biotin has an extremely high binding affinity for avidin; the dissociation constant is 10^{-15} M.[13] However, the binding of biotin to avidin is not completely specific to biotin; many biotin metabolites and structurally related compounds bind to avidin, though typically with lesser affinity.[13] For the high-performance liquid chromatography (HPLC)/avidin-binding assay used in our laboratory, biotin and biotin me-

Presented in part at Experimental Biology '96, Washington, DC, April 1996.
Supported by grants from the National Institutes of Health to DMM (DK36823).
Address reprint requests to Dr. D.M. Mock at Department of Pediatrics (GI), Arkansas Children's Hospital Research Institute, 800 Marshall Street, Little Rock, AR 72202-3591 USA
Received May 13, 1996; accepted July 1, 1996.

655 Avenue of the Americas, New York, NY 10010

0955-2863/96/$15.00
PII S0955-2863(96)00107-6

tabolites are analyzed in a two-step procedure.[1] First, the biotin analogs are separated by reversed-phase HPLC. Second, the biotin analogs in the collected fractions of the HPLC eluate are quantitated by measuring the binding to avidin coupled to horseradish peroxidase. This detection step is analogous to enzyme-linked immunoassays. Using this assay, we have quantitated biotin, bisnorbiotin, and biotin-*d,l*-sulfoxide in human serum and in human urine.[1,14] Besides these compounds, five unidentified avidin-binding compounds have been detected. We speculate that these compounds are either metabolites of biotin[15,16] or are structurally related compounds (*Figure 1*) that are not derived from biotin. In the experiments described here, we sought to determine whether certain naturally occurring compounds could account for the unidentified avidin-binding compounds or could interfere with avidin-binding assays.

Lipoic acid (6,8-thioctic acid, 1,2-dithiolane-3-pentanoic acid) and its metabolites are structurally similar to biotin. Both biotin and lipoic acid contain a valeric acid side chain and a sulfur-containing ring. Presumably as a result of these structural similarities, there are several functional similarities: (1) Lipoic acid binds to the biotin-binding site of avidin; however, the dissociation constant of avidin and *d,l*-lipoic acid is seven orders of magnitude weaker than that of biotin and avidin[17]; (2) avidin forms cross links between lipoic acid-containing acetyltransferase regions of the pyruvate dehydrogenase complex[18]; (3) biotinidase, the enzyme that cleaves biotinyllysine (biocytin), can cleave lipoyllysine[19]; and (4) the metabolic pathways of lipoic acid and biotin are similar.[20–22] Given the fact that biotin metabolites bind to avidin, one might predict that lipoate metabolites will also bind to avidin, though less tightly in analogy to lipoic acid itself. Whether lipoic acid will bind tightly enough to explain the unidentified compounds in human serum or urine or cause artifacts in avidin-binding assays is not clear from the theoretical considerations. The avidin binding of lipoic acid is much weaker than that of biotin; however, the plasma concentration of lipoic acid is about 100 fold higher than that of biotin.[23]

Urea and L-tryptophan might also interfere with an avidin-binding assay. Urea binds to avidin; the dissociation constant of the urea-avidin complex is 3.6×10^{-2} M.[13] Tryptophan is structurally similar to biotin; both possess a nitrogen-containing ring and a carboxylic acid side chain. However, tryptophan binding to avidin has never been reported. If tryptophan does bind to avidin, the number of related natural compounds that potentially could interfere with an avidin-binding assay would be substantial; L-tryptophan is an integral part of many peptides in body fluids. For the artificial derivatives L-tryptophan methyl ester, L-tryptophan amide, N-acetyl-D,L-tryptophan, and N-acetyl-L-tryptophan ethyl ester, either the amino group, the carboxyl group of the tryptophan moiety, or both are masked; thus the compounds are structurally similar to the naturally occurring tryptophan peptides. We propose that these peptide-mimicking compounds should provide information on the avidin affinity of tryptophan in peptides.

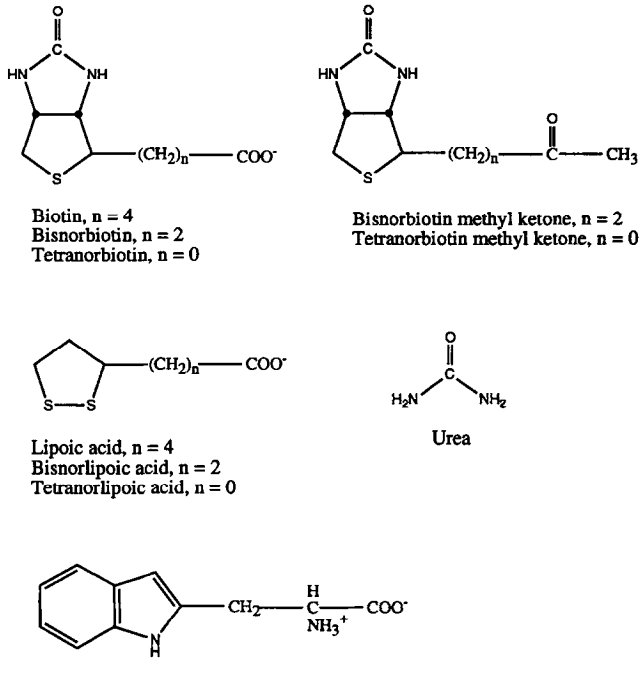

Figure 1 Structural formula of biotin analogs, lipoic acid analogs, urea, and tryptophan.

Methods and materials

Materials

Biotin, biocytin, *d,l*-lipoamide (6,8-thioctic acid amide), *d,l*-lipoic acid, L-tryptophan, and L-tryptophan amide hydrochloride were purchased from Sigma (St. Louis, MO, USA). Urea was purchased from Fisher Scientific (Fair Lawn, NJ, USA). L-Tryptophan methyl ester hydrochloride was purchased from Nutritional Biochemicals Corp. (Cleveland, OH, USA). N-Acetyl-D,L-tryptophan and N-acetyl-L-tryptophan ethyl ester were purchased from Aldrich (Milwaukee, WI, USA). Tetranorbiotin-*d,l*-sulfoxide, tetranorbiotin, biotin sulfone, tetranorbiotin methyl ketone, β-hydroxybiotin-*l*-sulfoxide, α,β-dehydrobisnorbiotin, biocytin-*d,l*-sulfoxide, biotin-*l*-sulfoxide, biotin-*d*-sulfoxide, bisnorbiotin methyl ketone, α,β-dehydrobiotin, and biotin methyl ester were synthesized chemically or biochemically; identities and purities were confirmed by extensive physico-chemical studies.[15,24] Bisnorbiotin was isolated from a subculture of the yeast *Rhodotorula rubra*.[14] Bisnorbiotin-*d,l*-sulfoxide was prepared according to Chastain et al.[25] Authentic standards of *l*(−)-lipoic acid, *d*(+)-lipoic acid, bisnorlipoic acid, β-hydroxybisnorlipoic acid, and tetranorlipoic acid were synthesized chemically or biochemically; identities and purities were confirmed by extensive testing.[20,21,26] For preparation of standard solutions of lipoic acid analogs, the crystalline compounds were dissolved in 95% ethanol under subdued light. Concentrations of lipoate analogs were quantitated by the absorbance at 330 nm in a Beckman DU 640 spectrophotometer (Beckman, Fullerton, CA, USA); molar extinction coefficients of 150 M^{-1} cm^{-1} (lipoic acid, β-hydroxybisnorlipoic acid, lipoamide), 160 M^{-1} cm^{-1} (bisnorlipoic acid), and 159 M^{-1} cm^{-1} (tetranorlipoic acid) were used.[26]

Instrumentation and chromatographic conditions

The HPLC instrumentation and chromatography method have been described previously.[14] The column temperature was kept at 25°C by immersion into a consistent temperature water bath (Matheson Scientific, Chicago, IL, USA). All chemicals used were

of HPLC grade or the highest purity available. The solvents were degassed under vacuum prior to use. Sample injection volume was 100 μL. The binary gradient (flow rate 1 mL/min) as reported by Chastain et al.[25] was modified as follows[1]: Starting from 100% solvent A (0.05% trifluoroacetic acid, wt/vol, pH 2.5), the proportion of solvent B (0.05% trifluoroacetic acid:acetonitrile, 1:1, by vol, pH 2.5) was linearly increased to 40% over 35 min. Solvent B was further increased in a linear fashion to 100% over 5 min and then cycled back to 100% of solvent A over 10 min. The 100% solvent A was continued for 5 min before the next injection was made. One-hundred and twenty μL of a mixture of 0.125 mol/L sodium hydroxide and 0.02 mol/L Hepes buffer (1.5:1, by vol) was added to each HPLC collection tube before collection. Thereby, the pH of the HPLC fractions was increased from 2.5 of the HPLC solution to 7.0 at the time of collection. This avoids oxidation artifacts.

Avidin-binding assay

The avidin-binding assay as described previously[1,27] was used for direct assessment of avidin-binding ability of all tested compounds and for determination of HPLC retention times of compounds with detectable avidin binding. Briefly, the sample to be assayed (pure solution or HPLC fraction) was incubated in a microtiter well with avidin-horseradish peroxidase. An aliquot was transferred into a second well precoated with biotinylated bovine serum albumin. After incubation, the plate was washed and *o*-phenylenediamine and H_2O_2 were added. The concentration of oxidized *o*-phenylenediamine was measured at 492 nm against a blank at 630 nm. Because the amount of avidin-horseradish peroxidase bound to the second plate decreases as the concentration of the avidin-binding substance (e.g. biotin) in the assayed sample increases, the optical density decreases with increasing biotin concentration in the sample.

For direct assessment of avidin affinities, standard solutions of pure compounds were used in the binding assay without prior HPLC separation. The concentrations in the standard solutions were chosen to span the ranges typically found in human serum and urine: Concentrations used were 0 to 28,000 pmol/L (biotin analogs), 0 to 114.4 nmol/L (lipoic acid analogs), 0 to 200 μmol/L (L-tryptophan), and 0 to 226.4 mmol/L (urea). *d,l*-Lipoic acid was also studied at a concentration of 1 μmol/L. The artificial tryptophan derivatives were assayed in the same concentration as used for L-tryptophan.

The relative binding to avidin was calculated as the ratio of the concentration of biotin (the reference compound) to the concentration of the compound that would produce the same optical density in the binding assay (eq. 1).

$$\text{Binding ratio} = (C_{\text{biotin}}/C_{\text{test compound}}) \quad (1)$$

where C_{biotin} (mol/L) = biotin concentration that produced the same optical density at 492 nm as the test compound did; $C_{\text{test compound}}$ (mol/L) = concentration of the test compound.

Hence, the affinity of biotin related to itself is 1.000. The calculated binding ratios represent the mean of at least three different pairs of concentrations; all concentrations were assayed in quadruplicate.

For determination of retention times of compounds on HPLC, standard solutions were injected, and the HPLC eluate was collected in fraction intervals of 0.25, 0.5, or 1.0 min as appropriate to resolve peaks as described previously.[1] Each HPLC fraction was analyzed using the avidin-binding assay. The fraction that contained the highest concentration of avidin-binding compound was reported as HPLC retention time. The avidin affinities of some compounds were too small at physiologic concentrations (see "Results") to be detected by the avidin-binding assay; thus their retention times could not be determined by the avidin-binding assay of the HPLC fractions. For those compounds, retention times were determined by measuring the absorbance of the HPLC eluate at 210 nm (biotin analogs), 280 nm (L-tryptophan), or 330 nm (lipoic acid analogs). The retention time of urea was determined by collecting the HPLC eluate in 1-min intervals, by derivatizing the urea with the addition of *p*-dimethylaminocinnamaldehyde,[28] and by measuring the absorbance at 533 nm.

The *l*- and *d*-isomers of biotin sulfoxide are not well-separated in this HPLC/avidin-binding system.[14] As in previous studies,[1] the isomers are referred to as "biotin sulfoxide." However, separation of the sulfoxide *d*- and *l*-isomers can be achieved using an anion exchange column.[25]

Results

Lipoic acid analogs

None of the tested lipoic acid analogs at physiologic concentrations showed detectable binding to avidin. Hence, these compounds are very unlikely to interfere in the avidin-binding assays for biotin or to produce an HPLC peak in the HPLC/avidin-binding assay. Moreover, all lipoic acid analogs except one were discretely separated from biotin analogs by HPLC (*Table 1*). The one exception was β-hydroxybisnorlipoic acid; this compound co-elutes with bisnorbiotin methyl ketone at 22.0 min (*Table 2*). At a concentration of 1 μmol/L, *d,l*-lipoic acid binding to avidin was not detectable.

L-*Tryptophan and tryptophan derivatives*

At physiologic concentrations, L-tryptophan binding to avidin was detectable. However, the binding ratio was small (3×10^{-6}). Though small, the binding to avidin is not attributable to analytical error. The mean coefficient of variation among quadruplicate analyses of 12 different concentrations in the avidin-binding assay of L-tryptophan was 7.5%. At concentrations less than 7.5 μmol/L, L-tryptophan did not produce detectable binding to avidin. The HPLC retention

Table 1 Retention times of lipoic acid analogs, urea, and tryptophan[a]

Compound	Retention time[b]
	min
Urea	5.0
d,l-Tetranorlipoic acid	18.3
β-Hydroxybisnorlipoic acid	21.7
L-Tryptophan	22.0
d,l-Bisnorlipoic acid	35.7
d,l-Lipoamide	42.2
l-Lipoic acid	44.3
d-Lipoic acid	44.3

[a]Separations were made on reversed-phase HPLC using a binary gradient (see text). Flow rate was 1.0 mL/min.
[b]Retention times were established measuring the absorbance of the HPLC eluate spectrophotometrically (330 nm for lipoic acid analogs, 280 nm for *L*-tryptophan). The eluate from urea injection was collected in 1-min intervals, derivatized by addition of *p*-dimethylaminocinnamaldehyde, and the absorbance was measured at 533 nm (see text).

Table 2 Retention times, detection limits, within-day variations of analyses, and avidin-binding ratios of biotin and biotin analogs[a]

Compound	Retention time	Detection limit	Within-day precision[b]	Binding ratio[c]
	min	*pmol/L*	%	
Tetranorbiotin-*d,l*-sulfoxide[d]	4.2	7,169	6.2 ± 0.9	0.003 ± 0.001
Tetranorbiotin[d]	6.9	—	—	n.d.
Biotin sulfone	7.0	55	10.2 ± 2.3	0.332 ± 0.025
Bisnorbiotin-*d,l*-sulfoxide	7.4	526	6.8 ± 3.0	0.038 ± 0.005
Tetranorbiotin methyl ketone[d]	8.4	906	5.4 ± 1.3	0.014 ± 0.003
β-Hydroxybiotin-*l*-sulfoxide	9.0	929	15.0 ± 3.1	0.023 ± 0.004
α,β-Dehydrobisnorbiotin	12.5	2,253	3.7 ± 2.8	0.009 ± 0.001
Biocytin-*d,l*-sulfoxide	13.5	129	11.4 ± 2.9	0.243 ± 0.008
Biotin-*l*-sulfoxide	13.5	60	11.9 ± 4.3	0.338 ± 0.015
Biotin-*d*-sulfoxide	13.9	47	6.3 ± 2.5	0.477 ± 0.037
Bisnorbiotin	15.5	47	7.4 ± 1.5	0.612 ± 0.088
Bisnorbiotin methyl ketone	22.0	25	6.9 ± 1.2	0.814 ± 0.031
Biocytin	22.5	29	7.3 ± 2.9	0.694 ± 0.069
α,β-Dehydrobiotin	27.5	39	18.7 ± 12.5	0.462 ± 0.066
Biotin	28.3	20	3.0 ± 1.4	1.000
Biotin methyl ester	43.0	12	9.3 ± 3.8	1.777 ± 0.403

[a]Separations were made on reversed-phase HPLC using a binary gradient (see text). Flow-rate was 1.0 mL/min.
[b]The coefficients of variation were calculated using standard concentrations in the linear slope of the calibration curve (see text). For each analog, the coefficient of variation was calculated from quadruplicate measurements of three to six different concentrations.
[c]Relative to *d*-biotin (=1.000).
[d]Because of the small affinity to avidin, the retention time was determined by measuring the absorbance of the HPLC eluate at 210 nm.

time of L-tryptophan (*Table 1*) was similar to that observed for bisnorbiotin methyl ketone.

The effect of L-tryptophan on the avidin-binding of biotin was further studied by measuring the biotin concentrations in mixtures of biotin (1500 pmol/L) and L-tryptophan (either 20 or 50 μmol/L) in the avidin-binding assay; the same concentration of biotin without L-tryptophan added served as a control. The addition of L-tryptophan increased the apparent concentration by about 24% compared to the control ($P < 0.002$, unpaired, two-tailed *t*-test).

For the four tryptophan derivatives, avidin binding was not detectable. Retention times for the artificial tryptophan derivatives were not determined.

Urea

At physiologic concentrations, the binding ratio of urea was small (1×10^{-9}). The mean coefficient of variation among quadruplicate analyses of 12 different concentrations in the avidin-binding assay was 6.1%. The limit of detection was 13.9 mmol/L. Urea did not co-elute with any of the available biotin analogs (*Tables 1* and *2*).

Biotin and biotin analogs

The HPLC retention times of 16 biotin analogs are shown in *Table 2*. Most analogs occur naturally.[15,16,24] Except as noted above, none of the available biotin analogs co-eluted with lipoic acid analogs, urea, or L-tryptophan. Biotin sulfone eluted at the same retention time as the unidentified peak number 1 in urine[14]; bisnorbiotin methyl ketone eluted at the same retention time as the unidentified peak number 3 in serum and urine.[1,14]

Chemical alterations of the biotin molecule leads to changes in the binding ratio (*Table 2*). The affinity of biotin methyl ester was higher than that of biotin, indicating that the carboxyl group of the biotin molecule is not essential for binding to avidin. Nevertheless, linkage of the carboxyl group to lysine in an amide bond (biocytin) reduced the binding ratio. If two carbon units or less were removed from the valeric acid side chain (α,β-dehydrobiotin, bisnorbiotin methyl ketone, and bisnorbiotin), the binding ratios were moderately decreased compared with biotin. Further side-chain shortening reduced the avidin-binding ratio substantially. The dehydrogenation of bisnorbiotin to α,β-dehydrobisnorbiotin reduced the binding ratio considerably. Similar weak binding was observed for tetranorbiotin-*d,l*-sulfoxide and tetranorbiotin methyl ketone. Sulfur oxidation in the thiophane ring also decreased the binding ratios of biotin analogs (biotin sulfoxides, biotin sulfone, biocytin-*d,l*-sulfoxide, bisnorbiotin-*d,l*-sulfoxide). For tetranorbiotin, avidin binding was not detectable.

The avidin affinities of biotin analogs were measured over a range of 0 to approximately 28,000 pmol/L. For optical densities between 0.2 and 0.7 calibration curves were linear. The concentration of each analog was always selected so that the optical density fell within this range. Linear regression analyses of the linear portions of the standard curves revealed regression coefficients of $r = 0.929$ to $r = 0.999$. For example, the binding ratios of biocytin-*d,l*-sulfoxide (analyzed concentration in parentheses) were 0.244 (978 pmol/L), 0.245 (652 pmol/L), 0.251 (435 pmol/L), and 0.231 (290 pmol/L). For most analogs, the lower limits of detection were less than 1000 pmol/L or were less than 100 pmol/L for several (*Table 2*). The mean (±SD) within-day precision of the assay (coefficient of variation) was 8.6% ± 4.2%.

Discussion

Although the fact that some compounds that are not metabolites of biotin bind to avidin is well established,[13] po-

tential interference in an avidin-binding assay for biotin has not been previously reported. In the present study, avidin binding was not detectable for lipoic acid or any of the lipoic acid analogs. Yet lipoic acid binds to avidin; the K_d of *d,l*-lipoic acid for avidin is 7×10^{-7} M.[13] We reconcile this apparent conflict as follows: If lipoic acid or its analogs bind to avidin at all, the binding is too weak to interfere with the subsequent binding to biotinylated albumin; alternatively, albumin binds lipoic acid (as it does fatty acids) in a manner that is competitive with lipoic acid binding to avidin. Specifically, we conclude either that lipoic acid is displaced from the biotin site on avidin by the biotin residues on albumin, or albumin binds lipoic acid or both.

Relative to lipoic acid, the structure of L-tryptophan resembles that of biotin less closely. Notwithstanding, L-tryptophan showed measurable binding to avidin, but lipoic acid did not. We speculate that the greater concentrations of L-tryptophan in the assay may account for the detectable avidin binding. Tryptophan concentrations in plasma[29,30] and urine[31] are in the μmolar range, whereas lipoic acid concentrations are in the nanomolar range.[23] To test this explanation we measured the binding of *d,l*-lipoic acid to avidin at 1 μmol/L (a concentration one order of magnitude below the concentration of L-tryptophan at which avidin binding was detectable). Although a suggestion of binding was present, the results were not statistically different from zero and hence we report that binding was not detected. The examination of greater concentrations was not possible because of the limit of *d,l*-lipoic acid solubility in aqueous solutions and because of the artifacts introduced in the assay by organic solvents used to prepare lipoic acid solutions of higher concentration.

Theoretically, L-tryptophan might interfere with the assay of bisnorbiotin-methyl ketone because the compounds co-elute on reversed-phase HPLC. Although the binding ratios of bisnorbiotin methyl ketone and biotin are both five orders of magnitude greater than that of L-tryptophan, the tryptophan at six orders of magnitude greater concentration measurably increased the apparent concentration of biotin when a mixture tryptophan and biotin was assayed. However, because the concentration of free L-tryptophan in human plasma is about 6 μmol/L,[29] which is approximately the detection limit of tryptophan in the avidin-binding assay, we anticipate the artifactual increase in apparent biotin will be less than 10%. L-Tryptophan amide, L-tryptophan methyl ester, N-acetyl-D,L-tryptophan, and N-acetyl-L-tryptophan ethyl ester are structurally similar to tryptophan peptides. None of these artificial tryptophan derivatives had measurable avidin binding. Therefore, these findings provide evidence that L-tryptophan peptides are not likely to interfere with avidin-binding assays for biotin.

Although the binding of urea to avidin is weaker ($K_d = 3.6 \times 10^{-2}$ M) than either that of *d,l*-lipoic acid or biotin,[13] some binding to avidin was detectable. We speculate that once again a difference in concentrations assayed was responsible for the apparent conflict. The urea concentrations used in the present studies were about nine orders of magnitude greater than those of the biotin analogs. Moreover, despite detectable avidin binding, we conclude that urea cannot interfere in the HPLC/avidin-binding assay, because urea is chromatographically separated from all biotin analogs.

In summary, none of the tested compounds is likely to interfere with the assay of biotin and its metabolites by an HPLC/avidin-binding assay. Even without prior HPLC separation of samples, none of the lipoic acid analogs, tryptophan analogs, or urea are likely to cause artifacts in avidin-binding assays. Even for the compounds that have detectable avidin binding, the binding is too weak to affect an avidin-binding assay at physiologic concentrations of the compounds. Likewise, this study provides evidence that none of the compounds studied could account for the unidentified avidin-binding compounds in plasma and urine previously reported.[1,14] We speculate that these avidin-binding substances are metabolites of biotin. In particular, we conclude it is not likely that lipoic acid analogs, tryptophan analogs, or urea at physiologic concentrations will interfere with avidin-biotin complex assays.

The findings of this study confirm findings of previous reports concerning the relative strength of the binding of biotin and biotin analogs to avidin (in particular to avidin coupled to horseradish peroxidase) and reinforce previous conclusions concerning errors that may be encountered in attempting to measure biotin in a mixture of biotin and biotin analogs. As reported previously,[13,14,32] the avidin affinities of biocytin, biotin sulfone, biotin sulfoxides, bisnorbiotin, or artificial biotin analogs are smaller than that of biotin. The present study adds new information on the relative avidin-binding abilities of a wide range of naturally occurring biotin metabolites. We found that all of the naturally occurring biotin analogs tested bind less tightly to avidin than biotin. Moreover, the presence of biotin metabolites is a potential source of error in avidin-binding assays for biotin if the metabolites are not removed (e.g., chromatographic separation) prior to assay. Likewise, quantitation of the various biotin metabolites by avidin-binding assays requires prior chromatographic separation and quantitation of each metabolite against a standard curve of the authentic metabolite. Finally, this study provides evidence that some analogs with very small avidin affinity will totally escape detection by avidin-binding assays. Studies with radiolabeled biotin (animal studies), stable isotope-labeled biotin (human studies), or pharmacologic amounts of biotin (human studies) will be required to investigate whether the biotin metabolites with small binding affinity are present at significant concentrations.

References

1 Mock, D.M., Lankford, G.L., and Mock, N.I. (1995). Biotin accounts for only half of the total avidin-binding substances in human serum. *J. Nutr.* **125,** 941–946

2 Livaniou, E., Evangelatos, G.P., and Ithakissios, D.S. (1987). Biotin radioligand assay with an ^{125}I-labeled biotin derivative, avidin, and avidin antibody reagents. *Clin. Chem.* **33,** 1983–1988

3 Bhullar, R.P., Lie, S.H., and Dakshinamurti, K. (1985). Isotope dilution assay for biotin. In *Biotin* (K. Dakshinamurti and H.N. Bhagavan eds.), p. 279–287, Academic Press Inc., San Diego, CA, USA

4 Chan, P.W. and Bartlett, K. (1986). A new solid-phase assay for biotin and biocytin and its application to the study of patients with biotinidase deficiency. *Clin. Chim. Acta* **159,** 185–196

5 Hood, R. (1979). Isotopic dilution assay for biotin: use of [^{14}C] biotin. In *Methods in Enzymology: Vitamins and Coenzymes* (D.B.

McCormick and L.D. Wright eds.), p. 279–287, Academic Press Inc., San Diego, CA, USA

6 Horsburgh, T. and Gompertz, D. (1978). A protein-binding assay for measurement of biotin in physiological fluids. *Clin. Chim. Acta* **82,** 215–223

7 Landman, A.D. (1976). A sensitive assay for biotin analogs and biotin-proteins. *Int. J. Vitam. Nutr. Res.* **46,** 310–313

8 Sanghvi, R.S., Lemons, R.M., Baker, H., and Thoene, J.G. (1982). A simple method for determination of plasma and urinary biotin. *Clin. Chim. Acta* **124,** 86–90

9 Yankofsky, S.A., Gurevitch, R., Niv, A., Cohen, G., and Goldstein, L. (1981). Solid-phase assay for *d*-biotin on avidin cellulose disks. *Anal. Biochem.* **118,** 307–314

10 Thuy, L.P., Sweetman, L., and Nyhan, W.L. (1991). A new immunochemical assay for biotin. *Clin. Chim. Acta* **202,** 191–198

11 Stein, J., Hahn, A., Lembcke, B., and Rehner, G. (1992). High-performance liquid chromatographic determination of biotin in biological materials after crown ether-catalyzed fluorescence derivatization with panacyl bromide. *Anal. Biochem.* **200,** 89–94

12 Przyjazny, A., Hentz, N.G., and Bachass, L.G. (1993). Sensitive and selective liquid chromatographic postcolumn reaction detection system for biotin and biocytin using a homogeneous fluorophore-linked assay. *J. Chromatogr.* **654,** 79–86

13 Green, N.M. (1975). Avidin. *Adv. Protein Chem.* **29,** 85–133

14 Mock, D.M., Lankford, G.L., and Cazin Jr., J. (1993). Biotin and biotin analogs in human urine: Biotin accounts for only half of the total. *J. Nutr.* **123,** 1844–1851

15 McCormick, D.B. and Wright, L.D. (1971). The metabolism of biotin and analogues. In *Metabolism of Vitamins and Trace Elements* (M. Florkin and E.H. Stotz eds.), p. 81–110, Elsevier Publishing Company, Amsterdam

16 McCormick, D.B. (1975). Biotin. *Nutr. Revs.* **33,** 97–102

17 Green, N.M. (1963). Avidin 3. The nature of the biotin-binding site. *Biochem. J.* **89,** 599–609

18 Hale, G., Wallis, N.G., and Perham, R.N. (1992). Interaction of avidin with the lipoyl domains in the pyruvate dehydrogenase multienzyme complex: Three-dimensional location and similarity to biotinyl domains in carboxylases. *Proc. R. Soc. Lond. B.* **249,** 247–253

19 Nilsson, L. and Ronge, E. (1992). Lipoamidase and biotinidase deficiency: evidence that lipoamidase and biotinidase are the same enzyme in human serum. *Eur. J. Clin. Chem. Clin. Biochem.* **30,** 119–126

20 Shih, J.C.H., Wright, L.D., and McCormick, D.B. (1972). Isolation, identification, and characterization of a lipoate-degrading pseudomonad and of a lipoate catabolite. *J. Bacteriol.* **112,** 1043–1051

21 Chang, H.-H., Rozo, M.L., and McCormick, D.B. (1975). Lipoate metabolism in Pseudomonas putida LP. *Arch. Biochem. Biophys.* **169,** 244–251

22 Spence, J.T. and McCormick, D.B. (1976). Lipoic acid metabolism in the rat. *Arch. Biochem. Biophys.* **174,** 13–19

23 Teichert, J. and Preiss, R. (1992). HPLC-methods for determination of lipoic acid and its reduced form in human plasma. *Int. J. Clin. Pharmacol.* **30,** 511–512

24 Kazarinoff, M.N., Im, W.-B., Roth, J.A., McCormick, D.B., and Wright, L.D. (1972). Bacterial degradation of biotin VI. Isolation and identification of β-hydroxy and β-keto compounds. *J. Biol. Chem.* **247,** 75–83

25 Chastain, J.L., Bowers-Komro, D.M., and McCormick, D.B. (1985). High-performance liquid chromatography of biotin and analogues. *J. Chromatogr.* **330,** 153–158

26 Shih, J.C.H., Williams, P.B., Wright, L.D., and McCormick, D.B. (1974). Properties of lipoic acid analogs. *J. Heterocycl. Chem.* **11,** 119–123

27 Mock, D.M. and Horowitz, P. (1990). A fluorometric assay for avidin-biotin interaction. In *Methods in Enzymology: Avidin-Biotin Technology* (M. Wilchek and E.A. Bayer eds.), p. 234–240, Academic Press Inc., San Diego, CA, USA

28 McCormick, D.B. and Roth, J.A. (1970). Specificity, stereochemistry, and mechanism of the color reaction between *p*-dimethylaminocinnamaldehyde and biotin analogs. *Anal. Biochem.* 226–236

29 Eynard, N., Flachaire, E., Lestra, C., Broyer, M., Zaidan, R., Claustrat, B., and Quincy, C. (1993). Platelet serotonin content and free and total plasma tryptophan in healthy volunteers during 24 hours. *Clin. Chem.* **39,** 2337–2340

30 Kojima, E., Kai, M., and Ohkura, Y. (1993). Determination of tryptophan in human serum by high-performance liquid chromatography with pre-column fluorescence derivatization using phenylglyoxal. *J. Chromatogr.* **612,** 187–190

31 Kema, I.P., Schellings, A.M.-J., Hoppenbrouwers, C.J.-M., Rutgers, H.M., de Vries, E.G.E., and Muskiet, F.A.-J. (1993). High performance liquid chromatographic profiling of tryptophan and related indoles in body fluids and tissues of carcinoid patients. *Clin. Chim. Acta* **221,** 143–158

32 Wright, L.D., Valentik, K.A., Nepple, H.M., Cresson, E.L., and Skeggs, H.R. (1950). Affinity of avidin for biocytin. *Proc. Soc. Exp. Biol. Med.* 273–274