Identification of biotin sulfone, bisnorbiotin methyl ketone, and tetrnorbiotin-\(d\)/-sulfoxide in human urine\(^1\)\(^-\)\(^3\)

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ABSTRACT  In previous studies using the HPLC and avidin-binding assay, five unidentified avidin-binding substances were observed in human urine. The present study investigated the identity of these substances. Urine was collected before and after intravenous administration of 18.5 \(\mu\)mol biotin to healthy adults. Unknown substances 1 and 3 were initially identified as biotin sulfone and bisnorbiotin methyl ketone, respectively, by coelution with authentic standards on HPLC. Identities were confirmed by thin-layer chromatography and by derivatization with \(p\)-dimethylaminocinnamaldehyde. As expected for biotin metabolites, the urinary excretion of biotin sulfone and bisnorbiotin methyl ketone increased with biotin administration. The urinary excretion of biotin sulfone increased 21-fold from 0.2 nmol/h before to 4.2 nmol/h after administration; the excretion of bisnorbiotin methyl ketone increased 130-fold from 0.4 to 51.8 nmol/h. At presumed steady state in free-living subjects \((n = 6)\), biotin sulfone and bisnorbiotin methyl ketone accounted for 3.6% and 7.9% of total biotin excretion, respectively. Traces of tetrnorbiotin-\(d\)/-sulfoxide were also identified by using thin-layer chromatography and derivatization with \(p\)-dimethylaminocinnamaldehyde. However, tetrnorbiotin-\(d\)/-sulfoxide was not detectable in urine by the HPLC and avidin-binding assay because this metabolite has weak avidin-binding affinity. We conclude that biotin sulfone and bisnorbiotin methyl ketone are present in measurable quantities in human urine; their quantitation should allow more accurate studies on human biotin metabolism and turnover.  


KEY WORDS  Biotin sulfone, bisnorbiotin methyl ketone, tetrnorbiotin-\(d\)/-sulfoxide, biotin, urine, human

INTRODUCTION

In our laboratory, biotin and biotin metabolites are routinely analyzed by using a two-step procedure \((1)\). First, the compounds are separated by HPLC. Second, the compounds in the HPLC fractions are quantitated with an avidin-binding assay. Using this assay, we quantitated biotin, bisnorbiotin, and biotin-\(d\)/-sulfoxide in human serum \((1)\) and urine \((2)\). In addition to these metabolites and biotin, we detected five unidentified avidin-binding compounds in serum or urine. Recently, we excluded the possibility that lipoic acid analogs, tryptophan analogs, and urea could account for these avidin-binding compounds \((3)\). We speculated that the unidentified substances were biotin metabolites on the basis of three observations.

1) The substances bind to avidin, suggesting a structural relation to biotin.
2) When radiolabeled biotin was administered parenterally to rats, radiolabeled compounds were detected in urine with HPLC retention times similar to those of several of the unknown avidin-binding substances in human urine \((4)\). In particular, a neutral ketone arising from administration of radiolabeled biotin to rats was reported previously by Lee et al \((5)\).
3) When radiolabeled biotin was administered to pigs, radiolabeled compounds with similar HPLC retention times were also detected \((6)\). The present study investigated the identity of these compounds in human urine.

SUBJECTS AND METHODS

Materials

Authentic standards of tetrnorbiotin-\(d\)/-sulfoxide, tetrnorbiotin, biotin sulfone, tetrnorbiotin methyl ketone, \(\beta\)-hydroxybiotin-\(d\)/-sulfoxide, \(\alpha,\beta\)-dehydrobisnorbiotin, biocytin-\(d\)/-sulfoxide, biotin-\(d\)/-sulfoxide, biotin-\(d\)/-sulfone, bisnorbiotin methyl ketone, \(\alpha,\beta\)-dehydrobiotin, and biotin methyl ester were synthesized chemically or biochemically; their identity and purity were confirmed by extensive physicochemical studies \((7, 8)\). Binosorbiotin and \([\text{\textsuperscript{14}C}]\)carbonyl bisnorbiotin were isolated from a subculture of the yeast Rhodotorula rubra \((2)\). Bisnorbiotin-\(d\)/-sulfoxide was prepared according to the method of Chastain et al \((9)\). Binosorbiotin and biocytin were purchased from Sigma Chemical Co \((St\) Louis\). \(\text{\textsuperscript{p}}\)-Dimethylaminocinnamaldehyde was purchased from Aldrich Chemical \((Milwaukie)\). All chemicals and solvents used were of HPLC grade or the highest purity available.

Experimental design

Timed urine samples were collected over 24 h from six healthy adults \((four\ women, two\ men)\). Thereafter, subjects

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received intravenous administration of 18.5 μmol (4.51 mg) biotin, after which one individual urine void was collected from each subject. To include the time of presumed maximal excretion of biotin and its metabolites (10), the urine samples were collected within 6 h after biotin administration. The duration of each urine collection was 1.9 ± 1.3 h (± SD); the midpoint of the sampling interval of the six urine samples was 3.2 ± 0.7 h after biotin administration. The protocol was approved by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences and informed written consent was obtained from all subjects.

The HPLC and avidin-binding assay

Biotin and known biotin metabolites were assayed with the HPLC and avidin-binding assay (2), with minor modifications as follows. The column temperature was kept at 25 °C by immersion in a constant temperature water bath (Matheson Scientific, Chicago). Depending on the concentration of biotin and its metabolites, the sample injection volume was adjusted between 200 and 1000 μL. The pH of the samples was adjusted to 2.5 by 6 mol HCl/L before injection. The binary gradient (flow rate 1 mL/min) as reported by Chastain et al (9) was modified as reported previously (1). Briefly, the HPLC fractions were buffered, evaporated, solubilized, and incubated in microtiter wells with avidin covalently bound to horseradish peroxidase. Aliquots were transferred onto a second plate pre-coated with biotinylated bovine serum albumin. After incubation, the plate was washed and o-phenylenediamine and hydrogen peroxide were added. The concentration of oxidized o-phenylenediamine was measured at 492 nm against a blank. The compounds in the fractions were quantitated in the avidin-binding assay by calibration against authentic standards. However, unidentified compounds 1–5 were calibrated against biotin standards, and the concentrations measured for unknown compounds 1 and 3 were corrected by using the previously reported avidin-binding affinities once these compounds were identified (3).

Thin-layer chromatography and derivatization with p-dimethylaminocinnamaldehyde

Identification of biotin metabolites by the HPLC and avidin-binding assay was confirmed by thin-layer chromatography (TLC) and subsequent derivatization with p-dimethylaminocinnamaldehyde. Urine as collected from two subjects after the administration of biotin was pooled; the volume was reduced ∼10-fold under vacuum at 80 °C. The pH of the concentrated urine was consistently between 6 and 7. The concentrated urine was centrifuged at 1560 × g for 10 min at 4 °C to remove any precipitates. Sample and standards were spotted on microcellulose TLC plates (250 μm, 20 × 20 cm; Eastman Kodak Company, Rochester, NY). Compound identification by TLC was made by using two different solvent systems: 1) 1-butanol: acetic acid:water (4:1:1, by vol) (11) and 2) 1-butanol alone (5). After being developed and dried, the plates were sprayed with p-dimethylaminocinnamaldehyde (12), which reacts with the less sterically hindered N-1 in the ureido portion of biotin and closely related analogs; an intense color with an absorption maximum at 533 nm is produced.

RESULTS

On the basis of retention times with the HPLC and avidin-binding assay, one urinary avidin-binding substance (previously denoted as unknown compound 1) was tentatively identified as biotin sulfoxide (7.0 min). A second compound (previously denoted as unknown compound 3) was identified as bisnorbiotin methyl ketone (22.0 min). Each was detectable in urine both before and after biotin administration (Figure 1). Although the relative contribution to the metabolite profile did not change strikingly with biotin administration (Figure 1), the urinary excretion of each compound increased substantially. The excretion of unknown compound 1 increased 21-fold from 0.2 ± 0.2 to 4.2 ± 1.3 nmol/h; the excretion of unknown compound 3 increased 130-fold from 0.4 ± 0.4 to 51.8 ± 19.4 nmol/h. Taken together, these observations provide strong evidence that unknown compounds 1 and 3 were biotin metabolites. Before biotin administration, biotin, bisnorbiotin, and biotin-dl-sulfoxide were also present in amounts similar to those reported previously (2). The urinary excretions of biotin, bisnorbiotin, and biotin-dl-sulfoxide increased 220-fold, 33-fold, and 86-fold, respectively, after biotin administration. The

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**FIGURE 1.** Chromatograms of urine samples before and after intravenous (iv) injection of 18.5 μmol (4.51 mg) biotin into an adult female. #1–#5, unknown metabolites #1 to #5; BSO, biotin-dl-sulfoxide; BNB, bisnorbiotin; BIO, biotin. Note the 40-fold change in the scales of the y-axes.
excretion of unknown compounds 2, 4, and 5 increased 25-, 145-, and 9-fold, respectively (Figure 1; note the 40-fold change in the scales of the y axes). These findings suggest that these compounds were also biotin metabolites; however, identification awaits further study.

TLC and derivatization with p-dimethylaminocinnamaldehyde were used to verify the identification of unknown compounds 1 and 3 as biotin sulfone and bisnorbiotin methyl ketone. Each unknown compound developed the appropriate color reaction with p-dimethylaminocinnamaldehyde. The $R_f$ value (ratio of the migration of the compound tested to the migration of the solvent front on the TLC plate) of the two unknowns and the respective standards matched for both TLC solvent systems (Table 1).

One additional urinary compound was detected by p-dimethylaminocinnamaldehyde development of TLC plates in postinfusion urine samples containing very large concentrations of biotin metabolites. The $R_f$ value matched that of tetranorbiotin-1-sulfoxide (Table 1). This unknown (ie, unknown compound 6) has not been detected previously in urine or serum with the HPLC and avidin-binding assay. This failure of detection is consistent with our observation that the avidin affinity of tetranorbiotin-1-sulfoxide is too small to allow detection by the avidin-binding assay at physiologic concentrations (3).

The absolute urinary excretion rates of biotin and its metabolites were determined in the 24-h urine samples collected before biotin administration (Table 2). Biotin sulfone and bisnorbiotin methyl ketone accounted for 3.6 ± 1.9% and 7.9 ± 5.8% of the total urinary biotin excretion, respectively. Tetranorbiotin-1-sulfoxide was not quantified because of weak binding to avidin.

**DISCUSSION**

In mammals, biotin is catabolized via $\beta$-oxidation of its valeric acid side chain and by oxidation of the sulfur atom in the thiophene ring (Figure 2). Side-chain degradation leads to the formation of bisnorbiotin (cleavage of two carbon units) or tetranorbiotin (cleavage of four carbon units) (13). Bisorbniotin methyl ketone and tetranorbiotin methyl ketone may be formed by nonenzymatic decarboxylation of the fairly unstable $\beta$-keto acids, which appear as intermediates of $\beta$-oxidation (8). The oxidation of the thioether sulfur in the thiophene portion of the molecule yields biotin-1-sulfoxide, biotin-d-sulfoxide, and biotin sulfone (7, 14). Moreover, the number of potential biotin metabolites is increased by the combination of side-chain degradation and sulfur oxidation. Cleavage of the imidazolidino and thiophene ring moieties by yielding carbon dioxide and urea does not play an important role in mammalian biotin metabolism (5). Biotin and the metabolites biotin-d-sulfoxide, biotin-1-sulfoxide, bisnorbiotin, and a neutral ketone have been identified in urine after administration of [14C]carbonyl biotin (4, 5).

Neither biotin sulfone nor bisnorbiotin methyl ketone have been reported previously as biotin metabolites in human urine. However, in total they account for ~12% of total urinary excretion of biotin plus metabolites. The presence of a neutral ketone in rat urine was reported previously (5); it seems likely that bisnorbiotin methyl ketone is this neutral ketone initially

<table>
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<th>TABLE 1</th>
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<td><strong>$R_f$ values of authentic standards of biotin metabolites and putative biotin metabolites on thin-layer chromatography (TLC)</strong></td>
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<tr>
<td><strong>Compound</strong></td>
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<tr>
<td>Unknown 1</td>
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<tr>
<td>Biotin sulfone</td>
</tr>
<tr>
<td>Unknown 3</td>
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<tr>
<td>Bisnorbiotin methyl ketone</td>
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<tr>
<td>Tetranorbiotin</td>
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<tr>
<td>Bisnorbiotin methyl ketone</td>
</tr>
<tr>
<td>Tetranorbiotin-d-sulfoxide</td>
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<tr>
<td>Unknown 6</td>
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<tr>
<td>Tetranorbiotin-l-sulfoxide</td>
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$1$ $R_f$, the ratio of the migration of the compound tested to the migration of the solvent front on the TLC plate.

$2$ Separation was on microcellulose plates (20 × 20 cm, 250 μm). Solvent 1 was 1-butanol:acetic acid:water (4:1:1, by vol); solvent 2 was 1-butanol.

$3$ The d- and l-isomers of tetranorbiotin sulfoxide were available as racemate only. Previous studies with d- and l-isomers of biotin sulfoxide and bisnorbiotin sulfoxide using similar solvents showed that the d-isomers migrated faster than the l-isomers (5). On the basis of these findings, we identified the tetranorbiotin sulfoxide present in human urine as the l-isomer.

<table>
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<th>TABLE 2</th>
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<tr>
<td><strong>Urinary excretion of biotin and biotin metabolites in healthy adults</strong></td>
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<tr>
<td><strong>Compound</strong></td>
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<tr>
<td>Biotin sulfone</td>
</tr>
<tr>
<td>Biotin-d/-sulfoxide</td>
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<tr>
<td>Bisnorbiotin</td>
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<tr>
<td>Bisnorbiotin methyl ketone</td>
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<tr>
<td>Biotin</td>
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<tr>
<td>Total biotin metabolites</td>
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$1$ $\pm$ SD; $n = 6$. Percentage excretion is in parentheses. Tetranorbiotin-l-sulfoxide was identified in urine but was not quantitated because of its small avidin affinity.

**FIGURE 2.** Structural formula of biotin analogues.
identified by Lee et al (5). Bisnorbiotin methyl ketone is a reasonable terminal product given what has been established previously concerning pathways for β-oxidation in general and of biotin in particular. Bisnorbiotin methyl ketone probably arises by spontaneous decarboxylation of the fairly unstable β-keto-biotin, which is a known intermediate in the β-oxidation of the valeric acid side chain (Figure 2) (5). On the basis of the pathway for β-oxidation of fatty acids (15), no further degradation of the side chain of bisnorbiotin methyl ketone is likely to occur in humans. Presumably as a consequence, bisnorbiotin methyl ketone accumulates to a detectable concentration in urine. We found no evidence of further degradation of bisnorbiotin methyl ketone via the oxidation of the ring sulfur (7). However, whether such a metabolite would retain sufficient avidin affinity to be detected by the HPLC and avidin-binding assay is not clear.

Biotin sulfone is also a terminal catabolite of biotin. The sulfur atom is fully oxidized, but theoretically biotin sulfone could be further degraded by β-oxidation of the valeric acid side chain. However, extensive studies on a pseudomonad able to utilize biotin as a sole source of carbon, nitrogen, and sulfur have shown that biotin sulfone has 1% relative conversion by β-oxidation compared with 80% for biotin-Δ-sulfone and 58% for biotin-7-sulfone (16).

Tetranorbiotin-7-sulfone is also probably a terminal catabolite in the metabolism of biotin in humans. This compound has a fully degraded side chain and, except for oxidation yielding tetranorbiotin sulfone, could only be further degraded by ring cleavage. Sulfur oxidation (as present in tetranorbiotin-7-sulfone) is an obligatory step in ring cleavage (7). Ring cleavage would result in formation of 5-sulfoxymethyl-2-oxo-4-imidazolidinone-acetic acid and ultimately in degradation to carbon dioxide and urea. However, little or no ring cleavage occurs in mammals (5); thus, one might expect tetranorbiotin sulfones (Δ- or l-isomers) to be terminal metabolites in mammals. Before the observation presented here, side-chain degradation in the biotin molecule further than to bisnorbiotin has not been reported for humans. The novel identification of a tetranorbiotin derivative clearly shows that side-chain catabolism to tetranorbiotin is possible. However, this study did not allow quantitative assessment of this pathway because the avidin affinity of tetranorbiotin-7-sulfone is too small to allow quantitation of the metabolite in the HPLC and avidin-binding assay.

Data on vitamin activities of the new biotin metabolites in mammals are not available. On the basis of structural changes and analogy to bisnorbiotin (no vitamin activity), biotin-Δ-sulfone (no vitamin activity), and biotin-7-sulfone (no vitamin activity), we speculate that none of the new metabolites retain vitamin activity. However, it is possible that they might interfere with the renal reabsorption of biotin. It has been shown that biocytin inhibits the uptake of biotin by basolateral membrane vesicles from rat kidney (17). In analogy, the intestinal transport of biotin is reduced in the presence of biotin analogs (11, 18).

In agreement with our previous findings in serum (1) and urine (2), additional peaks (unknown compounds 2, 4, and 5) were detected with the HPLC and avidin-binding assay. These unknown substances did not coelute with any of our biotin analogs. We conclude that the unknowns are not any of those biotin analogs; however, on the basis of increased excretion after biotin administration, we speculate that unknown compounds 2, 4, and 5 are indeed biotin metabolites. The presence of side-chain degraded metabolites of biotin (bisnorbiotin, bisnorbiotin methyl ketone, and tetranorbiotin-7-sulfone) in urine indicates that the intermediates of β-oxidation (eg, α,β-dehydro-, β-hydroxy-, and β-keto compounds) must be produced intracellularly during the metabolism of biotin. These compounds might account for the unknowns. On the basis of apparent relations between structure and avidin-binding affinity, we speculate that the unknown compounds are biotin metabolites with side chains no shorter than three carbon atoms.

The skilful technical assistance of GM Heird in the HPLC and avidin-binding assay is gratefully acknowledged.

REFERENCES