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

Janos Zempleni, Donald B McCormick, and Donald M Mock

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 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 tetrarnorbiotin-\(\gamma\)-sulfoxide were also identified by using thin-layer chromatography and derivatization with \(p\)-dimethylaminocinnamaldehyde. However, tetrarnorbiotin-\(\gamma\)-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. Am J Clin Nutr 1997; 65:508–11.

KEY WORDS Biotin sulfone, bisnorbiotin methyl ketone, tetrarnorbiotin-\(\gamma\)-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-\(\gamma\)-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 tetrarnorbiotin-\(\gamma\)-sulfoxide, tetrarnorbiotin, biotin sulfone, tetrarnorbiotin methyl ketone, \(\beta\)-hydroxybiotin-\(\gamma\)-sulfoxide, \(\alpha,\beta\)-dehydrobisnorbiotin, biocytin-\(\gamma\)-sulfoxide, biotin-\(\alpha\)-sulfoxide, biotin-\(\delta\)-sulfoxide, 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). Biotin and \(\text{[14C]}\)carbonyl bisnorbiotin were isolated from a subculture of the yeast Rhodotorula rubra (2). Biotin-\(\gamma\)-sulfoxide was prepared according to the method of Chastain et al (9). Biotin and biocytin were purchased from Sigma Chemical Co (St Louis). \(p\)-Dimethylaminocinnamaldehyde was purchased from Aldrich Chemical (Milwaukee). 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 precoated 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-dimethylaminoncinamaldehyde

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 miccelulose 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 sulfone (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
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 tetrnorbiotin-\( l \)-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 tetrnorbiotin-\( l \)-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. Tetrnorbiotin-\( l \)-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 thiophane ring (Figure 2). Side-chain degradation leads to the formation of bisnorbiotin (cleavage of two carbon units) or tetrnorbiotin (cleavage of four carbon units) (13). Bisorbiotin methyl ketone and tetrnorbiotin 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 thiocarbon Sulfur in the thiophane portion of the molecule yields biotin-\( l \)-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 imidazolidone 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-\( l \)-sulfoxide, bisnorbiotin, and a neutral ketone have been identified in urine after administration of \([^{14}C] \)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 \( \approx 12% \) 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

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<th>Table 2</th>
<th>Urinary excretion of biotin and biotin metabolites in healthy adults</th>
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<td>Compound</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 \( \mu \)m). 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 tetrnorbiotin sulfone were available as racemate only. Previous studies with \( d \)- and \( l \)-isomers of biotin sulfone and bisnorbiotin sulfone using similar solvents showed that the \( d \)-isomers migrated faster than the \( l \)-isomers (5). On the basis of these findings, we identified the tetrnorbiotin sulfone present in human urine as the \( l \)-isomer.
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 con-
centration in urine. We found no evidence of further degrada-
tion 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 sulfoxide is also a terminal catabolite of biotin. The
sulfur atom is fully oxidized, but theoretically biotin sulfoxide
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 sulfoxide has 1% relative conversion by
β-oxidation compared with 80% for biotin-d-sulfoxide and
58% for biotin-l-sulfoxide (16).

Tetranorbiotin-l-sulfoxide is also probably a terminal catab-
olite in the metabolism of biotin in humans. This compound
has a fully degraded side chain and, except for oxidation
yielding tetranorbiotin sulfoxide, could only be further degraded
by ring cleavage. Sulfur oxidation (as present in tetranorbiotin-
l-sulfoxide) is an obligatory step in ring cleavage (7). Ring
cleavage would result in formation of 5-sulfoxyethyl-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
sulfoxides (d- or l-isomers) to be terminal metabolites in mam-
mals. Before the observation presented here, side-chain degrada-
tion 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 catab-
olism to tetranorbiotin is possible. However, this study did not
allow quantitative assessment of this pathway because the
avidin affinity of tetranorbiotin-l-sulfoxide 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-d-
sulfoxide (no vitamin activity), and biotin-l-sulfoxide (no vi-
tamin 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 intesti-
nal 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)
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-l-sulfoxide) in urine indicates
that the intermediates of β-oxidation (e.g. α,β-dehydro-, β-hy-
droxy-, 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 un-
known compounds are biotin metabolites with side chains no
shorter than three carbon atoms.

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binding assay is gratefully acknowledged.

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