Biotin accounts for less than half of all biotin and biotin metabolites in the cerebrospinal fluid of children \(^1\--\(^3\)

Anna Bogusiewicz, Shawna L Stratton, Dale A Ellison, and Donald M Mock

**ABSTRACT**

**Background:** Biotin is likely transported into cerebral spinal fluid (CSF) via one or more specific transporters. Concentrations of biotin in CSF measured by using modern analytic techniques that are specific for biotin and biotin metabolites have not previously been reported.

**Objectives:** We aimed to accurately measure the concentration of biotin and major biotin metabolites, biotin sulfoxide (BSO) and bisnorbiotin (BNB), in the CSF of children.

**Design:** Concentrations of biotin were determined initially as total avidin-binding substances (TABS) in CSF obtained by lumbar puncture from 55 children. Biotin, BSO, and BNB were quantitated by HPLC and an avidin-binding assay in CSF samples from a subset of 11 children.

**Results:** Concentrations of TABS in CSF averaged 1.6 nmol/L with substantial variability (SD = 1.3 nmol/L). CSF concentrations of biotin and biotin analogs varied widely, but substantial amounts of BSO were detected in every sample. Biotin accounted for 42 ± 16%, BSO for 41 ± 12%, and BNB for 8 ± 14% of the total. It was surprising that the molar sum of biotin, BSO, and BNB on average was >200-fold the TABS concentrations from the same CSF sample. Using several analytic approaches, we found no masking of detection, nor did we find degradation of biotin or BSO. Gel electrophoresis and streptavidin Western blot detected several biotinylated proteins in CSF.

**Conclusions:** Biotin appears to be bound to protein covalently, reversibly, or both, and this binding likely accounts for the increase in detectable biotin after HPLC. Protein-bound biotin may play an important role in biotin nutriture of the brain. *Am J Clin Nutr* 2008;88:1291–6.

**INTRODUCTION**

Biotin is a water-soluble vitamin that plays an essential biochemical role in all mammals. In humans, biotin acts as a coenzyme for 5 carboxylases: propionyl-coenzyme A (CoA) carboxylase, 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase, and 2 isoenzymes of acetyl-CoA carboxylase. These biotin-dependent carboxylases catalyze vital reactions in important metabolic pathways involving gluconeogenesis, fatty acid synthesis, catabolism of branched-chain amino acids, and metabolism of some neurotransmitters (1, 2). Mammals fill their biotin requirement through diet and perhaps through absorption of the biotin produced by the normal microflora of the intestine (3, 4).

As do all other mammalian cells, neurons require biotin for normal function. In humans, biotin deficiency causes neurologic symptoms including seizures, hypotonia, developmental delay, ataxia, visual problems (eg, optic atrophy), and sensorineural hearing loss. Children with inborn errors of biotin metabolism, such as holocarboxylase synthetase deficiency (infantile multiple carboxylase deficiency) and individual carboxylase deficiencies (eg, propionyl-CoA carboxylase deficiency), are treated with high doses of biotin for months or years. Some respond well to this therapy; others do not. For example, in the case of holocarboxylase synthetase deficiency, defects in binding affinity (Michaelis constant, \(K_m\)) respond well to biotin therapy, whereas defects in maximum velocity (\(V_{max}\)) have a more limited response. To optimize therapy, biotin must reach the central nervous system, which could be indirectly assessed by measuring the biotin concentration in the cerebrospinal fluid (CSF).

For proper interpretation, a normal range of biotin content in CSF should be established. In previous studies of the concentration of biotin in CSF, biotin was analyzed by using an avidin-binding assay without metabolite separation (5). Investigations in our group’s laboratory have provided evidence that the presence of avidin-binding substances in addition to biotin can confound the ability of avidin-binding assays to quantitate biotin in serum, plasma, and urine (6, 7).

The present study sought to accurately measure the concentration of biotin and 2 other major biotin metabolites, bisnorbiotin (BNB) and biotin sulfoxide (BSO), in the CSF of children by using HPLC and an avidin-binding assay (HPLC/avidin-binding assay). The present study also investigated the potential mechanism of an observed discrepancy between total avidin-binding substances (TABS) measured by using a direct avidin-binding assay and the molar sum of biotin and the 2 metabolites measured by HPLC avidin-binding assay.

**SUBJECTS AND METHODS**

**Study population characteristics**

CSF was obtained from children undergoing lumbar puncture for routine diagnostic purposes and was cultured for routine infective causes in our group’s laboratory. The present study also investigated the potential mechanism of an observed discrepancy between total avidin-binding substances (TABS) measured by using a direct avidin-binding assay and the molar sum of biotin and the 2 metabolites measured by HPLC avidin-binding assay.

1 From the Departments of Biochemistry and Molecular Biology (AB, SLS, and DMM), Pathology (DAE), and Pediatrics (DAE and DMM), University of Arkansas for Medical Sciences, Little Rock, AR.

2 Supported by grant no. R37 DK 36823 from the National Institutes of Health.

3 Reprints not available. Address correspondence to DM Mock, Department of Biochemistry and Molecular Biology #516, University of Arkansas for Medical Sciences, Little Rock, AR 72205. E-mail: mockdonaldm@uams.edu.

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microbiological diagnosis and subjected to cell counting to assess inflammation and neoplasia. CSF remaining after these clinically indicated procedures was provided in a de-identified form for the present research project. Inclusion criteria included sterility on bacterial culture and no evidence of inflammation or neoplasia based on cell count. Samples from children with nutritional disorders and diseases with predictable malnutrition (eg, inflammatory bowel disease) were excluded. The subjects’ ages ranged from 9 mo to 18 y. Samples were stored at −70 °C until they were assayed.

The study was approved by the Institutional Review Board of the University of Arkansas for Medical Sciences. The Institutional Review Board approved the study as exempt; the samples used were pre-existing and stripped of all identifiers except diagnosis, and therefore, the Board did not require informed consent.

Quantitation of biotin and biotin metabolites in cerebrospinal fluid

TABS were quantitated in the CSF of 55 children by using a previously described sequential solid-phase avidin-binding assay (8). In CSF from a subset of 11 children, biotin and biotin metabolites were separated by HPLC using a C18 reversed-phase column as previously described (9). Briefly, 1-mL aliquots of CSF were fractionated by using HPLC. The HPLC fractions collected at specific retention times were determined to contain specific metabolites by comparison to retention times of authentic radiolabeled biotin metabolites synthesized as previously described (9). Our group (6, 9, 10) has reported that, for both unlabeled biotin and [3H]-biotin, recovery by HPLC is 95%. Biotin, BNB, and BSO were the only significant avidin-binding substances detected in CSF (see Results); they were quantitated according to the manufacturer’s recommendations.

Electrophoretically resolved proteins were electroblotted onto a polyvinylidifluoride membrane for 1 h at 30 V. The membrane was blocked, washed, and incubated with streptavidin-peroxidase conjugate (Roche Diagnostics, Indianapolis, IN) as previously described (13). The membrane was developed by using an immunopure metal-enhanced diaminobenzidine substrate kit (Thermo Scientific, Waltham, MA) according to the manufacturer’s recommendations.

Confirmation of identification of biotin metabolites

To confirm that an early-eluting avidin-binding substance at the BSO fraction was indeed BSO—rather than biotin bound either covalently or reversibly to another molecule, eg, a protein or an oligopeptide, which could result in possible co-elution with the BSO fraction—we re-chromatographed the avidin-binding substance from the BSO fraction. The HPLC fractions were collected and assayed for avidin-binding substances. The identities of the detected avidin-binding substances were inferred from a comparison of their retention times with those established for radiolabeled biotin and biotin metabolites (9).

Evaluation of the possibility that biotin is bound reversibly or covalently to macromolecules

To assess whether biotin is bound to macromolecules, ultrafiltration of CSF pooled from 10 children was performed by centrifugation for 1 h at 5000 × g in 10-kDa devices (Centricon; Millipore, Milford, MA). Biotin was quantitated in the ultrafiltrate and retentate by using an avidin-binding assay as described previously (8). Total biotin covalently bound to macromolecules was measured by releasing biotin with the use of acid hydrolysis. Released biotin was quantitated by using an avidin-binding assay as described previously (8, 11).

Analysis of cerebrospinal fluid proteins

CSF proteins were resolved electrophoretically by using an adaptation of the method of Lewis et al (12) and were stained with colloidal Coomassie blue stain (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations.

Electrophoretically resolved proteins were electroblotted onto a polyvinylidifluoride membrane for 1 h at 30 V. The membrane was blocked, washed, and incubated with streptavidin-peroxidase conjugate (Roche Diagnostics, Indianapolis, IN) as previously described (15). The membrane was developed by using an immunopure metal-enhanced diaminobenzidine substrate kit (Thermo Scientific, Waltham, MA) according to the manufacturer’s recommendations.

Statistical analysis

Central tendency is expressed as the mean. Variability is expressed as the SD with the use of STATVIEW software (version 5.0.1; SAS Inc, Cary, NC).

RESULTS

Results for CSF samples from 55 children along with diagnostic classification are shown in Table 1. The mean (±SD) concentration of TABS was 1.6 ± 1.3 nmol/L. TABS values ranged from undetectable (≤0.022 nmol/L) to 5.9 nmol/L.

On the basis of our group’s previous studies of the chemical composition of the avidin-binding substances in plasma and
The cerebrospinal fluid (CSF) samples from 11 subjects were analyzed for total avidin-binding substances (TABS) with the use of an avidin-binding assay. For 9 of the 11 subjects, the total of biotin, BNB, and BSO measured by HPLC/avidin-binding assay was at least 50-fold the TABS value. By adding biotin independently identified and quantified as [3H]-biotin to CSF before the avidin-binding assay, we further evaluated whether an underestimation of biotin in CSF as measured by direct avidin-binding assay could be the result of a potential masking effect. The measured increase in biotin by the avidin-binding assay accounted for 100% of the added biotin, which provides evidence against masking.

We also evaluated the possibility that factors or microbes present in CSF may have degraded or taken up biotin during storage in the clinical laboratory, thus artifically decreasing measured biotin concentration and producing the observed biotin metabolites. When [3H]-biotin was incubated in CSF and then subjected to HPLC, the recovery of total radioactivity was 97%, which provides evidence against biotin loss. No conversion of the radiolabeled biotin to biotin metabolites was observed.

### TABLE 1

<table>
<thead>
<tr>
<th>Diagnosis of subject</th>
<th>TABS (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute lymphoblastic leukemia (n = 23)</td>
<td>0.6 ± 0.9</td>
</tr>
<tr>
<td>Lymphoblastic lymphoma (n = 3)</td>
<td>0.2</td>
</tr>
<tr>
<td>Fever (n = 6)</td>
<td>1.7</td>
</tr>
<tr>
<td>Pneumonia (n = 4)</td>
<td>1.2</td>
</tr>
<tr>
<td>Respiratory syncytial virus infection (n = 2)</td>
<td>0.5</td>
</tr>
<tr>
<td>Seizure (n = 3)</td>
<td>2.1</td>
</tr>
<tr>
<td>Respiratory distress (n = 2)</td>
<td>3.0</td>
</tr>
<tr>
<td>Apnea (n = 2)</td>
<td>4.5</td>
</tr>
<tr>
<td>Burkitt’s lymphoma (n = 2)</td>
<td>0.2</td>
</tr>
<tr>
<td>Spina bifida with CNS shunt (n = 1)</td>
<td>0.5</td>
</tr>
<tr>
<td>Urinary tract infection (n = 1)</td>
<td>1.6</td>
</tr>
<tr>
<td>Upper respiratory infection (n = 1)</td>
<td>4.1</td>
</tr>
<tr>
<td>Cerebral palsy or CNS shunt (n = 1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Headache (n = 1)</td>
<td>0.5</td>
</tr>
<tr>
<td>Gastroesophageal reflux (n = 1)</td>
<td>1.7</td>
</tr>
<tr>
<td>Head injury (n = 1)</td>
<td>0.7</td>
</tr>
<tr>
<td>Bronchopulmonary dysplasia (n = 1)</td>
<td>3.0</td>
</tr>
<tr>
<td>Mean</td>
<td>1.6 ± 1.3</td>
</tr>
</tbody>
</table>

1 CNS, central nervous system. Total samples, n = 55.
2 Range: 0.022–5.9.
3 ± SD (all such values).

### TABLE 2

<table>
<thead>
<tr>
<th>Subject diagnosis</th>
<th>Biotin</th>
<th>Biotin sulfoxide</th>
<th>Bisnorbiotin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Percentage of total</td>
<td>Concentration</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>0.33</td>
<td>31.0</td>
<td>0.41</td>
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<tr>
<td>Acute lymphoblastic leukemia</td>
<td>18.0</td>
<td>69.5</td>
<td>6.63</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>4.64</td>
<td>15.1</td>
<td>15.7</td>
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<tr>
<td>Acute lymphoblastic leukemia</td>
<td>18.8</td>
<td>38.3</td>
<td>26.5</td>
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<tr>
<td>Acute lymphoblastic leukemia</td>
<td>29.1</td>
<td>34.9</td>
<td>53.0</td>
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<tr>
<td>Acute lymphoblastic leukemia</td>
<td>42.1</td>
<td>51.7</td>
<td>372</td>
</tr>
<tr>
<td>Burkitt’s lymphoma</td>
<td>0.39</td>
<td>28.8</td>
<td>0.35</td>
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<tr>
<td>Lymphoblastic lymphoma</td>
<td>29.9</td>
<td>59.5</td>
<td>17.3</td>
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<tr>
<td>Lymphoblastic lymphoma</td>
<td>24.7</td>
<td>30.7</td>
<td>26.3</td>
</tr>
<tr>
<td>Head injury</td>
<td>22.9</td>
<td>39.5</td>
<td>21.6</td>
</tr>
<tr>
<td>Bronchopulmonary dysplasia</td>
<td>160</td>
<td>58.0</td>
<td>108</td>
</tr>
<tr>
<td>Mean</td>
<td>66.4</td>
<td>41.6</td>
<td>58.9</td>
</tr>
<tr>
<td>SD</td>
<td>126</td>
<td>16.2</td>
<td>108</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.33</td>
<td>15.1</td>
<td>0.35</td>
</tr>
<tr>
<td>Maximum</td>
<td>421</td>
<td>69.5</td>
<td>372</td>
</tr>
</tbody>
</table>

1 The cerebrospinal fluid (CSF) samples from 11 subjects were analyzed for total avidin-binding substances (TABS) with the use of an avidin-binding assay. The HPLC fractions of the CSF samples from these 11 persons were analyzed for biotin and biotin analogs with the use of an avidin-binding assay. Data for TABS and biotin analogs represent the mean of ≥3 determinations analyzed against the biotin standard curve. Biotin sulfoxide and bisnorbiotin were analyzed against biotin sulfoxide and bisnorbiotin standard curve, respectively.
observed by HPLC, which provides evidence against artifactual degradation.

Re-chromatography of the BSO fraction resulted in the recovery of 99% of the avidin-binding substance. The substance continued to elute at the retention time for BSO. This observation provides evidence that the avidin-binding substance is indeed BSO, rather than biotin bound to a larger molecule.

We also tested the possibility that significant amounts of biotin in CSF are bound to macromolecules. By centrifugal ultrafiltration, we separated free biotin from any biotin that may be bound to macromolecules with molecular weight of ≥10 kDa. Concentrations of biotin in the ultrafiltrate and retentate were quantified with the use of an avidin-binding assay. The ratio of retentate value to ultrafiltrate value was 1.2 ± 0.05. These data suggest that only ≈20% of biotin could be bound to macromolecules. However, this approach does not rule out the possibility that biotin may be bound to macromolecules in a way that masks detectability by an avidin-binding assay (eg, bound inside the protein).

We further evaluated the possibility that biotin is bound to macromolecules and released during acidic HPLC. One CSF sample underwent acid hydrolysis, and the total avidin-binding substances increased ≈30-fold. This increase accounted for most, but not all, of the 90-fold increase observed for this particular sample after HPLC. This finding suggested that acid hydrolysis and acidic HPLC release biotin from macromolecules (possibly CSF proteins) to which biotin was bound covalently or reversibly in a location in the macromolecule that was not accessible to avidin.

We visualized proteins present in CSF and further assessed whether any of these proteins were biotinylated. As expected, there were many proteins, and they appeared in large amounts in CSF (Figure 1). Several biotinylated proteins bands were detected, the most prominent of which were at 12–15, 55, 60–65, 70–75, and >100 kDa (Figure 2). It is interesting that the location of the protein band with the highest concentration in CSF coincided with a band of biotinylated proteins.

**DISCUSSION**

These studies provide evidence that direct avidin-binding assays underestimate biotin concentrations in CSF of children and that acidic HPLC separation of biotin and biotin analogs substantially increases the detectable concentrations of both biotin and BSO. One explanation for this finding is the possibility that biotin and biotin metabolites bound to proteins are released during the HPLC process.

In CSF from 55 children, we found that the average TABS concentration was 1.6 nmol/L (range: 0.02–5.9 nmol/L). The concentrations of TABS in CSF reported in the present study are approximately twice those reported by Lombard and Mock (14) for fasting concentrations of TABS in plasma from children. As reported by those authors using the same avidin-binding assay as used in the present study, the average TABS concentration detected was 0.83 nmol/L (range: 0.56–1.08 nmol/L). Anagnostouli et al (5) reported biotin concentrations in CSF from adults using a similar avidin-binding assay. Those investigators found mean concentration of biotin in CSF to be 0.56 ± 0.31 nmol/L. Our findings with respect to the concentrations of biotin in the CSF of adults are similar to but moderately greater than those reported by Anagnostouli et al. However, they found that the concentrations of biotin in adult CSF are only one-third of those reported for fasting concentrations of biotin in serum from adults. Such a difference may be related to differences in subjects (eg, timing of samples or biotin intake) or assay differences.

Our finding that the concentrations of TABS in CSF are approximately twice those reported for plasma is similar to the finding of Lo et al (15) in rats, obtained by using an avidin-binding assay. Those investigators reported that biotin concentrations in rat CSF are 2.5 times those in serum concentrations.

We found substantially different results when CSF was subjected to HPLC under acidic conditions and then assayed. The total of biotin plus the 2 major metabolites, BNB and BSO, was an average of 240 times the concentrations of TABS. For an avidin-binding assay to measure biotin specifically, biotin must be the only avidin-binding metabolite in the CSF. The present studies provide evidence that it is not. The equilibrium-binding constant for avidin varies among the biotin metabolites (16). BSO and BNB have binding affinities to avidin that are less than the affinity of biotin and hence that are likely to be less efficiently detected. As a consequence, these metabolites will be underestimated when measured in a mixture with biotin and quantitated against a biotin standard curve (7). However, on the basis of our group’s previous reports (6, 9, 17), the true concentrations of BSO and BNB were only ≈3-fold the amounts found in analyses against a biotin standard curve. This 3-fold increase cannot explain the 240-fold concentration observed in the current study.

Approximately half of the TABS found is attributable to biotin metabolites rather than to biotin per se. In contrast to the relative concentrations of metabolites in the plasma and urine of adults (9,
observed in urinary metabolites did not confirm this possibility; drug induction generally favored an increase in BNB over one in BSO (21).

Further assessment of the cause of the large observed variability is limited by study design. Other unknown factors may have affected subject heterogeneity. For example, subjects may have received biotin supplementation, but this information was not available because of the use of de-identified samples. If subjects were supplemented, variability in biotin and metabolites concentrations in CSF may be the consequence of greater biotin intake (23, 24).

Our investigation of the mechanism leading to underestimation of the amount of true biotin in CSF by direct avidin-binding assay yielded evidence to support the following 4 conclusions. First, masking effects did not occur. Second, artificial conversion of the radiolabeled biotin to biotin metabolites (either by degradation or uptake by microbes) did not occur. Third, incorrect identification of biotin metabolites did not occur. Fourth, artificial conversion of biotin sulfone to a more-oxidized metabolite such as biotin sulfone did not occur.

We hypothesize that the increase in measurable biotin and biotin metabolites arises, in large part, from the release of biotin from biotin-binding macromolecules such as proteins. Ultrafiltration experiments suggested that most of the biotin is bound in a way that is not accessible to avidin (eg, in the interior of the macromolecules) and, as such, that it is “hidden” from the assay. Acid hydrolysis experiments produced a 30-fold increase of the biotin concentration in CSF, which is consistent with a mechanism in which most of the biotin in CSF is bound to the interior of macromolecules.

In summary, these data provide evidence that CSF contains amounts of biotin, BSO, and BNB that substantially exceed the amount detected by a direct avidin-binding assay. The present studies provide evidence that the release of biotin that was reversibly or covalently bound to protein explains most of the increases in detectable biotin after HPLC separation of biotin and biotin analogs. We conclude that most of the biotin in CSF is bound to protein.

The authors’ responsibilities were as follows—DAE (Study Coordinator): collected all clinical samples; AB, SLS, and DMM: designed the study; AB and SLS: performed all laboratory measurements and techniques; AB: drafted the manuscript and prepared the tables; SLS: prepared the figures; all authors: edited the manuscript; and DMM (Principal Investigator): was responsible for the final version of the manuscript. None of the authors had a personal or financial conflict of interest.

REFERENCES