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Short communication

Determination of biotin levels in cerebrospinal fluid samples

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1. Introduction

Biotin is a vitamin of the B-complex group. In humans, this substance functions as the cofactor of four carboxylating enzymes, known as carboxylases, which are involved in the fixation of carbon dioxide in various substrates in vivo [1]. Carboxylation reactions are component steps of several metabolic processes, including glyconeogenesis, fatty acid synthesis and catabolism of several branched-chain amino acids. Unlike many bacteria, humans cannot synthesize biotin and, therefore, derive the vitamin from food intake, possibly from biotin synthesizing gastrointestinal microflora and, finally, from the in vivo turnover of the biotin-containing carboxylases [2]. The latter process is essential for providing an adequate biotin supply to human cell machinery [3]. For biotin to be recycled, however, carboxylases must first be degraded proteolytically to biocytin, i.e. biotinyl-ε-lysine, or to short biotinyl-peptides. Biotinidase is the enzyme that cleaves the biotinyl-moiety from the lysyl residue, thus liberating biotin and allowing its recycling [4]. Errors in biotin turnover in humans are often manifested by neurological problems. Oral administration of pharmacological doses of biotin is usually effective and can reverse most of the symptoms, including the neurological ones, if biotin deficiency is diagnosed early and the vitamin is given regularly and life-long [5]. It is therefore, important to maintain an adequate biotin status in human tissues and systems, especially in the nervous system.

Cerebrospinal fluid biotin levels may reflect the vitamin status in the nervous system and can,

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therefore, reveal important information, especially for patients with neurological disorders. Taking this into consideration, we have developed, evaluated, and present here a radioligand assay for measuring biotin in cerebrospinal fluid samples (CSF samples) by properly modifying an assay previously developed in our laboratory for determining human serum biotin levels [6,7].

2. Experimental

2.1. Assay reagents

Radiotracer: This was a mixture of mono- and di- ^{125}I-labelled biotinyl-tyramines synthesized as previously described [8]. The radiotracer working solution (350 kCi mol^{-1}) was prepared in phosphate buffer (pH 6.5; 0.5 M), kept at 4°C for 2 months in lyophilized form, and reconstituted before running the assay.

Biotin standards: These were prepared in phosphate buffer (pH 6.5; 0.04 M) containing 0.72 g l^{-1} NaCl and 0.35 g l^{-1} bovine serum albumin. A series of standard solutions were used in the assay containing 0, 15, 40, 80, 160, 320, 640 or 960 ng of biotin per litre. Freshly redistilled and sterilized water was used for preparing the phosphate buffer and consequently the biotin standard solutions. The biotin standard solutions were kept at −35°C for 1 year.

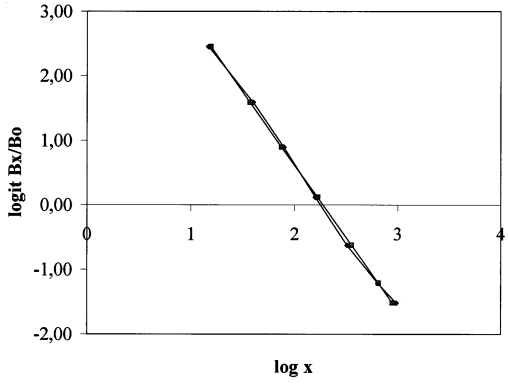

Fig. 1. Shape of assay standard curve (◆, point to point curve; □, corresponding least square linear line).

Avidin: This was used as the assay binding protein. The avidin working solution (10 μg l^{-1}) was prepared in a 1.6 g l^{-1} aqueous solution of human serum albumin, as a stabilizing protein, kept at 4°C for 2 months in lyophilized form, and reconstituted before running the assay.

Protein source reagent: This was biotin-free, biotinidase inhibited pooled human serum. The above reagent was obtained after treating pooled human serum (tested for absence of HIV and hepatitis viruses) with activated charcoal (10% w/v, overnight incubation under stirring, 4°C), removing charcoal with centrifugation (4000 × g, 30 min, 4°C) and filtration through paper filter, and then incubating the filtrate with a 4 mM sodium p-hydroxymercuribenzoate suspension (2 ml per 100 ml of filtrate) for 1 h at 37°C.

Precipitation reagent: This was a 200 g l^{-1} polyethylene glycol 6000 solution in phosphate buffer (pH 7.4; 0.05 M).

2.2. Assay protocol and standard curve

A total of 100 μl of biotin standard solutions or unknown CSF samples, 200 μl of the radiotracer working solution and 50 μl of the avidin working solution were added in a 3-ml polystyrene tube and incubated for 30 min at room temperature. Then, 50 μl of the protein source reagent and 2 ml of the precipitation reagent were added to each tube and mixed thoroughly. The tubes were then centrifuged (3000 × g) for 15 min at room temperature. After discarding the supernating liquid, the radioactivity of the precipitate was measured. In the blank tubes, water was added instead of the avidin working solution.

A linear assay standard curve was obtained (Fig. 1) by plotting B_x/B_0 versus standards concentration on logit/log scale, using the following linear equation:

$$\text{logit}\ (B_x/B_0) = \ln\ \{(B_x/B_0)/[1-(B_x/B_0)\} = \alpha + \beta\ \log x,$$

where B_0 is the fraction of the radiotracer bound to avidin when the zero biotin standard is added, B_x is the fraction of the radiotracer bound when the remaining (other than zero) biotin standards are added, x is the biotin concentration, α is the

Table 1
Assay precision

Intra-assay		Interassay	
Biotin (ng l^{-1})[a]	CV[b]	Biotin (ng l^{-1})	CV
47 ± 6	12.8	47 ± 9	19.1
102 ± 8	7.8	102 ± 10	9.8
204 ± 12	5.9	204 ± 15	6.9

[a] Mean $\pm$ SD:

$$SD = \sqrt{\frac{\sum d^2}{2n}},$$

where: d, difference between duplicates in each pair of the samples assayed; n, total number of pairs (10, and 24 pairs of each sample were assayed for determining intra- and interassay precision, respectively).

[b]

$$CV = \frac{(SD)}{mean} 100.$$

y-intercept, and β is the slope of the assay standard curve.

2.3. Assay analytical characteristics

The intra- and interassay precision were determined using control CSF samples at three different concentrations, as previously described [7]. Assay accuracy was determined by adding known biotin amounts to or, diluting with zero standard, CSF samples of low or high biotin concentration, respectively, and calculating the % recovery [7]. Assay precision and accuracy data are presented in Tables 1 and 2, respectively. The assay sensitivity, evaluated as previously described [7,9] was estimated to be less than 10 ng l^{-1}.

2.4. Assay clinical evaluation

The assay was clinically evaluated by assaying in duplicate CSF samples obtained from 46 presurgical individuals with no evidence of neurological disorders (control group) and from eight epileptic patients under long-term therapy with certain anticonvulsant agents. Data obtained were expressed as mean values $\pm$ SD. Prior to the assay, the CSF samples were properly treated by adding 20 μl of a 4 mM sodium p-hydroxymercury-benzoate (p-HMB) suspension per ml of sample and incubating for 1 h at 37°C [7]. The samples were kept at -70°C for 6 months.

3. Results and discussion

Biotin concentrations measured in human CSF samples could provide important insight information concerning the vitamin status in the nervous system of human individuals, especially of patients with neurological disorders. However, little information can be found in the literature regarding biotin levels in human cerebrospinal fluid. The only report, to our knowledge, on human CSF

Table 2
Assay accuracy

Recovery of exogenous biotin added to human CSF[a]			Linear recovery of endogenous biotin in diluted human CSF[b]		
Biotin added to CSF sample (ng l^{-1})	Measured biotin values (ng l^{-1})	%Recovery	Sample dilution	Measured biotin values (ng l^{-1})	%Recovery
20	61	91	–	204	–
320	378	103	1:1	96	94
640	756	110	1:3	54	106
–	–	–	1:7	29	114

[a] Known amounts of exogenous biotin were added to a pooled human CSF sample containing low endogenous biotin concentration (47 ng l^{-1}).

[b] Pooled human CSF sample of high endogenous biotin concentration (204 ng l^{-1}) was diluted with assay zero standard.

biotin content is that of Baker et al. [10], which mentions the passage of biotin, among several B-vitamins, from blood to cerebrospinal fluid in normal subjects. This limited information may be due, at least to some extent, to the lack of a commercially available assay-kit for measuring biotin levels in biological fluids, or, even of a reliable, sensitive and convenient analytical method, especially designed for assaying CSF samples.

Ligand assays have improved biotin analytical methodology over the last few years, due to their reliability, sensitivity, and ease of performance. Moreover, ligand assays are especially suitable for assaying such complex and delicate samples as biological fluids. These assays have replaced, therefore, the tedious and sometimes non-specific microbiological methods, which were previously used for determining biotin in human biological samples (mainly blood serum, plasma and urine). However, no ligand assay has been especially developed for measuring biotin in human cerebrospinal fluid. Thus, aiming at measuring biotin in human CSF samples, we have properly modified a radioligand assay previously developed in our laboratory for serum biotin [6,7] and evaluated its performance in determining CSF biotin levels.

The radiotracer used in the assay was a high specific activity mixture of mono- and di- ^{125}I-labelled biotinyl-tyramine. Full details about the radiotracer synthesis and purification were previously described [8]. To avoid radiotracer cleavage, the CSF samples to be assayed were properly pretreated, so as to inhibit any biotinidase activity [7].

The biotin standard solutions used in the assay were prepared in sterilized water. When unsterilized water was used, the standard solutions of low biotin concentrations (15–40 ng l^{-1}) responded as if they had had higher biotin concentrations than the real ones. Moreover, the apparent biotin concentrations seemed to differ greatly from batch to batch standard preparation. A possible explanation is that tiny amounts of biotin, derived from microorganisms that synthesize biotin, could be present in distilled water [11]. These trace amounts of biotin were not detectable in biotin standard solutions of higher concentrations, but their presence was noticeable in standards containing as low biotin concentration as 1.5 pg/100 μl. This problem was finally circumvented by using water sterilized immediately after its redistillation.

Avidin was used as the assay binding protein, due to its well known specificity and high affinity for the biotin molecule. Avidin was preferred to streptavidin [12], since its assay performance was excellent and its cost is considerably lower than that of streptavidin. No problems of non-specific binding were observed, despite the high pI value and the saccharic-moiety present in the avidin molecule, probably due to the type of the assay developed (not solid-, but liquid-phase assay).

In order to separate radiotracer bound to avidin from the corresponding free fraction, 2 ml of a PEG precipitating solution, in combination with 50 μl of biotin-free, biotinidase inhibited pooled human serum, as a protein source, were added to each assay tube, either containing standards or unknown samples. A total of 25, 50 75 and 100 μl of the latter reagent were used in preliminary experiments, which revealed that 50 μl were adequate for complete precipitation of the radiotracer bound to avidin.

In order to test its capability of determining cerebrospinal fluid biotin in normal and abnormal cases, the assay was applied to the measurement of biotin levels in human CSF samples obtained from 46 control individuals and eight epileptic patients under medication with certain anticonvulsants.

Biotin concentrations determined in CSF samples of the control subjects ranged from 50 to 321 ng l^{-1}. The vast majority of the control subjects had CSF biotin concentrations ranging between 50 and 150 ng l^{-1} (Fig. 2), whereas the mean CSF biotin value was 126 ± 53 ng l^{-1}.

Epileptic patients under long-term therapy with certain anticonvulsant agents (e.g. carbamazepine) were used in the assay clinical evaluation, since they were previously reported to have significantly lower serum biotin levels than apparently healthy individuals [13] and were, therefore, expected to show lower CSF biotin levels as well. The biotin concentrations determined in the CSF samples of

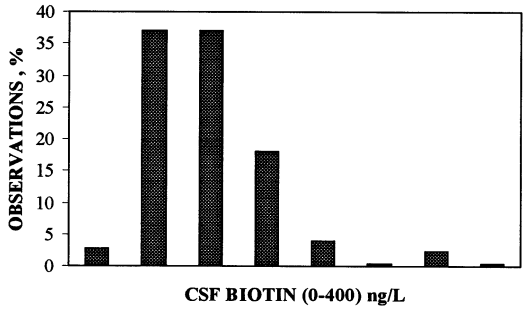

Fig. 2. Biotin concentration determined in cerebrospinal fluid (CSF) samples obtained from 46 neurologically normal individuals (control group). First from left bar corresponds to individuals (%) with CSF biotin of 0–50 ng l^{-1}, second bar corresponds to individuals (%) with CSF biotin of 50–100 ng l^{-1}, etc.

the epileptic patients were indeed $\sim 40\%$ lower than those measured in the control subjects and this verifies the capability of our method to determine low biotin levels in abnormal CSF samples.

In conclusion, this study refers to the development and the analytical evaluation of a radioligand assay for determining biotin in human cerebrospinal fluid. The assay developed was applied to measuring biotin in CSF samples obtained from both neurologically normal individuals and epileptic patients under anticonvulsant medication, who were expected to have reduced CSF biotin levels. To our knowledge, the above method is the only one reported so far in the literature, which has been designed for measuring biotin in CSF samples.

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