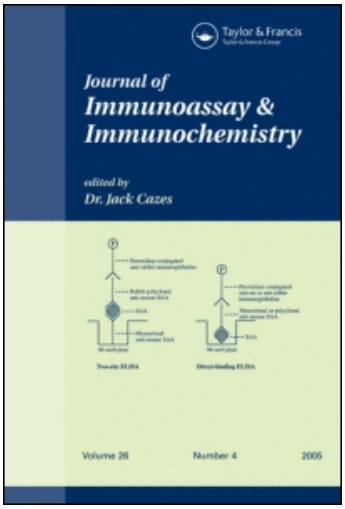

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INDIRECT ENZYME-LINKED METHOD FOR DETERMINING BIOTIN IN HUMAN SERUM

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ABSTRACT

An indirect enzyme-linked assay was developed for quantifying biotin concentrations in human sera. Biotin standard solutions or unknown samples are preincubated with streptavidin-conjugated horseradish peroxidase (streptavidin-HRP) and added to plates coated with biotinylated bovine IgG (B-IgG_b). The concentration of the streptavidin-HRP is such that the streptavidin binding sites are sufficient to bind apparently all the biotin present in samples, whereas, the remaining sites are inversely proportional to the amount of biotin in analysed sample. These sites could subsequently interact with the immobilized B-IgG_b providing signal. The assay demonstrated dynamic range 5 to 640 ng/L, detection limit 2 ng/L, intra- and interassay C.V., 1.6-3.9 % and 3.7-7.2 % respectively, recovery 100-114% and linear recovery 90-117%. Serum biotin determined: healthy individuals 66 to 600 ng/L, pregnant women ($\geq$ 36weeks) 60 to 360 ng/L, and patients under chronic haemodialysis 0.56 to 1.62 µg/L. The method described is among those few which have been experimentally evaluated for their capabilitity of assessing biotin in human sera.

(*Key words*: biotin, biotinylated bovine IgG, streptavidin-HRP, indirect enzyme-linked assay, human serum samples)

Address for correspondence

INTRODUCTION

Biotin is a water-soluble vitamin of the B-complex group, also known as vitamin H. It serves as an essential coenzyme for four mammalian carboxylases which catalyze several important reactions in metabolic pathways (1). Renewed interest in biotin stems from the accumulating reports of various biotin-responsive inborn errors of metabolism which have been connected with malfunction syndromes or symptoms, mainly neurological and cutaneous ones (2-7). However, it has not been completely elucidated whether biotin level fluctuations in human can be used as a reliable indicator for diagnosing or monitoring the above syndromes, or whether these fluctuations are associated with the development of other diseases. On the other hand, it is known that most of these symptoms can be cured or prevented by supplementation with biotin in pharmacological doses.

Most reports on the determination of biotin were traditionally based on microbiological assays using *Ochromonas danica* (8) or *Lactobacillus plantarum* (9). In recent times, several binding assays with improved analytical characteristics have been employed for the assessment of biotin concentrations. Mostly, they rely on the use of labile radioisotopic preparations (10-15) or require complicated and time consuming protocols (16-21). Other assays, are either rapid but less sensitive (22-25) or have high sensitivity but their applicability to biological fluids is questionable due to interfering substances (26,27). Nevertheless, only few biotin assays have been specially designed and evaluated for clinical laboratory use. To our knowledge, the most validated one in several population groups (28-30) is the radioligand assay developed by our research group (13). However, this assay has the disadvantages connected with utilization of radioactive tracers.

In the present study, we developed a simple and rapid enzyme-linked assay (ELA), especially designed for routine determination of biotin in human serum, using commercially available reagents. The assay method has been evaluated by assessing biotin in three specially selected reference population groups (apparently healthy individuals,

pregnant women, patients under chronic haemodialysis) with known (13) serum biotin levels (normal, low, and high, respectively) to validate the reliability of the proposed method.

MATERIAL AND METHODS

Materials

All reagents were analytical grade. The water used for standard preparation was distilled and sterilized. Flat-bottom 96-well microtitre plates were from Costar (Cambridge, MA). Polystyrene test tubes were from Vive Tubes Co., (Athens, Greece). Microplate Shaker EAS 2/4 was from Labinstruments (A-5082 Grodig, Austria). The streptavidin-horseradish peroxidase conjugate, bovine serum albumin, bovine IgG, 2,2'-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) diammonium salt (ABTS), d-biotin, sodium p - hydroxymercuribenzoate and Tween 20 were from Sigma Chemical Co., (St. Louis, MO). Sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) was purchased from Pierce Chemical Co., (Rockford, IL). Dimethyl-sulfoxide (DMSO) and all other chemicals were from Merck-Schuchardt (Darmstadt, Germany).

Buffers and Solutions

Coating buffer was a sodium carbonate/bicarbonate buffer (0.05 M) pH 9.2, and blocking solution was 1% (w/v) BSA in sodium bicarbonate buffer (0.1 M) pH 8.5, containing 0.05% (w/v) sodium azide. Washing solution A was a phosphate buffer (0.04 M) pH 6.5, containing 7.2 g/L of NaCl, and washing solution B was the washing solution A containing 0.05% (v/v) Tween 20. Assay buffer was the washing solution A containing 3.6% (w/v) BSA. Substrate solution was ABTS, 1 mg/mL, in citrate/phosphate buffer (0.1M) pH 4.4, containing 0.003 % H_2O_2. Biotinidase inhibitor was a sodium p-hydroxymercuribenzoate suspension, 37 g/L.

Biotin Standards

Biotin standard solutions ranging from 5 to 640 ng/L were prepared from a 100 µg/L bulk biotin solution diluted with an appropriate volume of assay buffer. The zero standard contained no biotin. Biotin standards could be stored at -35°C up to two years.

Samples

Serum samples were obtained from clinically well-characterized individuals: 68 apparently healthy subjects, 35 pregnant women at the final trimester of pregnancy and 40 patients with chronic renal failure undergoing haemodialysis treatment. Biotinidase in sera was inhibited by adding 20 µL of the inhibitor suspension per mL of serum and incubating for 1h at 37°C (31). Serum aliquots of three hundred µL were transferred in 3-mL polystyrene tubes and stored at -35°C until assayed.

Preparation of Biotinylated Bovine IgG (B-IgG_b)

Biotin was covalently coupled to bovine IgG (IgG_b) using sulfosuccinimidyl-6- (biotinamido)hexanoate (NHS-LC-biotin) as follows: ten µL of a 100 mg/mL solution of NHS-LC-biotin in DMSO were added slowly under mixing to 1 mL of a IgG_b solution (1 mg/mL) in sodium bicarbonate buffer (0.3M) pH 8.5, providing a 3:1 molar ratio of NHS-LC-biotin to IgG_b amino groups (one molecule of IgG_b is supposed to contain 95 free amino groups). The reaction was allowed to proceed for 90 min at room temperature, followed by extensive dialysis of the mixture at 4°C against sodium bicarbonate buffer (0.1M) pH 8.5, containing 9 g/L of NaCl and 0.1% (w/v) sodium azide, to remove any trace of unreacted NHS-LC-biotin. The biotinylated IgG_b could be stored as stock solution up to 12 months at 4°C.

Assay Protocol

All incubations were at room temperature. The microtitre plate wells, except blanks, were coated with 100 µL/well of a 2 µg/mL solution of B-

IgG_b in coating buffer. Coating buffer was added to the blank wells. After overnight incubation, the coating solution was discarded, the plates were washed once with washing solution A to remove unbound protein, and blocking solution was added to each well and incubated for 1h. Then, the plates were emptied and washed three times with washing solution B. The plates were then incubated (30 min) with 50 µL of streptavidin-HRP conjugate (20 ng/mL in assay buffer), and 50 µL of standard biotin solutions or serum samples, which had been pre-incubated separately in 3-mL polystyrene test tubes for 15 min. The microtitre wells were then emptied, washed three times with washing buffer B, and incubated with 100 µL/well of the substrate solution for 45 min in the dark. Color development was read at 405 nm, with a 490 nm reference filter, in an ELISA reader (Dynatech MR 5000, Germany). Measurements were performed in duplicate and a standard ELA curve was included in each plate.

Standard Curve

$B/B_o.100$ is plotted against concentration of biotin standards and the best curve between the points is drawn. A typical standard curve, from which the concentration of biotin in serum samples is determined, is shown in Fig. 1.

RESULTS

Assay Optimization

Microplates were coated with 100 µL of B-IgG_b solutions and incubated for 18h at room temperature. The binding capacity of the immobilized B-IgG_b was evaluated using the zero biotin standard, and following the assay protocol described in Materials and Methods. Different B-IgG_b preparations were tested, which were obtained using 6:1, 3:1, 1.5:1, 0.6:1 molar ratios of NHS-LC-biotin to the IgG_b amino groups. As shown in Figure 2, all immobilized B-IgG_b were capable of effective binding to the streptavidin-HRP detection reagent. In all cases, the

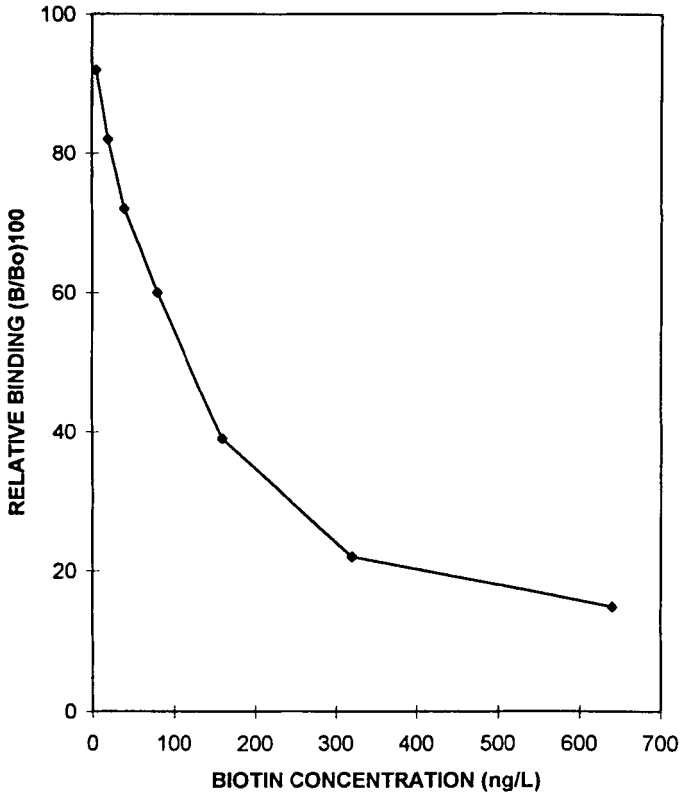

FIGURE 1. Biotin ELA standard curve (mean % B/B_0, n=10).
B_o represents the fraction of streptavidin-HRP immobilized on the B-IgG_b coated plates when zero standard biotin is added, and B the fraction of streptavidin-HRP immobilized in the presence of biotin standards (5-640 ng/L) or serum biotin.

relative rate of change in binding signal followed an initial rapid increase before reaching a maximum at higher coating concentration, then gradually falling. We selected B-IgG_b (3:1) for further investigation. Solutions of this B-IgG_b with increasing concentrations from 0 to 10 mg/L, were immobilized at various pH, ranging from 7.0 to 9.6, using previously reported in the literature coating buffers, such as Tris buffer, pH 8.0 or

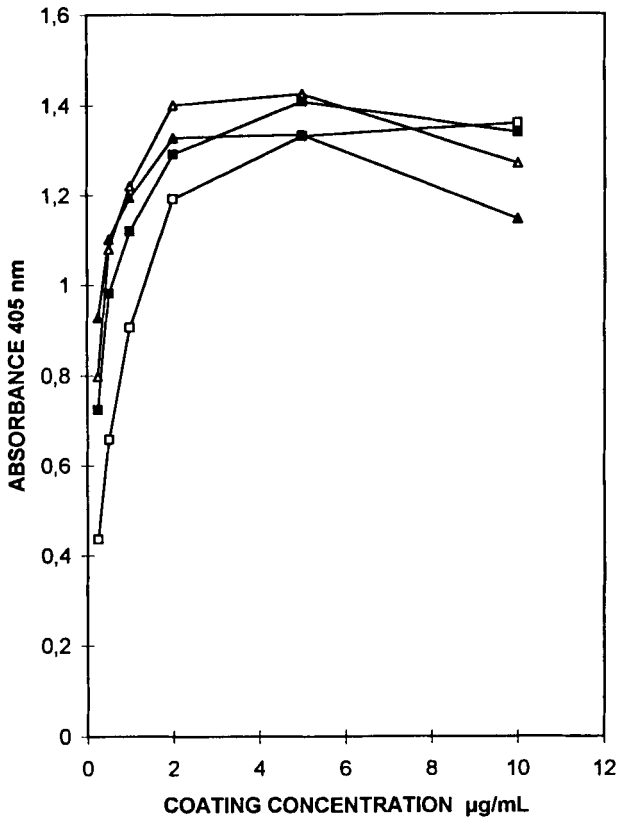

FIGURE 2. Absorbance reported as a function of different coating concentrations of biotinylated bovine IgG. Biotinylation of bovine IgG was performed as described in Materials and Methods using different molar ratios of NHS-LC-biotin to IgG_b. Lines with open square, closed square, open triangle and closed triangle represents 0.6:1, 1.5:1, 3:1 and 6:1 molar ratios respectively.

7.2, phosphate buffer, pH 8.0 or 7.0, and bicarbonate buffer, pH 9.2 or pH 9.6 (32). As revealed, optimum absorbance value was obtained using a 2 mg/L concentration of B-IgG_b in bicarbonate buffer, pH 9.2 in comparison with other coating buffers tested (data not shown). Sealed B-IgG_b coated microtitre plates, stored in coating buffer at 4^0C, were sufficiently stable for assay use up to 2 months.

Streptavidin-HRP solutions, with conjugate concentrations ranging from 10 to 50 ng/mL, were tested. The solution providing optimal standard curve characteristics (detection limit and dynamic range) was that of 20 ng/mL and this was selected as the assay working solution. Streptavidin-HRP was added to the B-IgG_b coated microtitre plates after 15 min preincubation with biotin standard solutions or assay samples. Preincubation may last up to 30 min without affecting results. The assay non-specific signal, probably caused by non-specific binding of the streptavidin-HRP congugate into uncoated microwells, was <3% of the B_0 value (absorbance of the zero standard).

Analytical Variables

Detection limit

The detection limit, defined as the least amount of analyte significantly different from zero at 95% confidence limits (i.e. mean for 12 replicates of zero standard - 2SD) was 2 ng/L (33).

Intra-assay and interassay precision

The intra-assay precision of the standard curve was determined from five standard curves run in duplicate during five days (34). The standards were run simultaneously with control serum samples containing 112-548 ng of biotin per litre. The intra-assay CV averaged 4.9% over the range 5 to 640 ng/L of the standards and 2.6% over the range of the control sera (Table 1). The interassay precision of the standard curve was determined from 10 standard curves run in duplicate over a period of 10 weeks (34). The standards were run simultaneously with control serum samples containing 151-574 ng of biotin per litre. The interassay coefficient of variation averaged 8.8% over the range of the standards tested and 5.7% for the control serum (Table 2).

TABLE 1

Intra-assay precision

Standards			Sera		
Biotin added (ng/litre)	$(B/B_0)100$	CV[b]	Endogenous biotin (ng/litre)	CV	n[c]
5	94.2 ± 2.3[a]	2.4	548 ± 8.5	1.6	10
20	87.6 ± 3.4	3.8	287 ± 11.1	3.9	10
40	75.0 ± 3.0	4.0	112 ± 2.7	2.4	10
80	63.8 ± 3.0	4.7			
160	42.2 ± 2.2	5.1			
320	23.8 ± 1.6	6.9			
640	16.8 ± 1.3	7.7			

[a]Mean ± standard deviation

The standard deviation (SD) of the $(B/B_0)100$ and sera is calculated from the differences between replicates run on each assay.

$SD=\sqrt{\frac{\sum d^2}{2n}}$ where d=difference between duplicates and n the number of pairs.

[b]$CV=\frac{(SD)}{mean}100$

[c]n=the number of control sera samples tested

TABLE 2

Interassay precision

Standards			Sera		
Biotin added (ng/litre)	$(B/B_0)100$	CV[b]	Endogenous biotin (ng/litre)	CV	n[c]
5	95.0 ± 1.9[a]	2.0	574 ± 21.3	3.7	10
20	88.9 ± 2.8	3.1	271 ± 16.8	6.2	10
40	75.8 ± 4.4	5.8	151 ± 10.8	7.2	10
80	62.8 ± 3.6	5.7			
160	39.6 ± 5.7	14.4			
320	23.7 ± 2.8	11.9			
640	16.4 ± 3.0	18.2			

[a]Mean ± standard deviation

The standard deviation (SD) of the $(B/B_0)100$ is calculated from the mean of the duplicate determinations done on each assay.

[b]$CV=\frac{(SD)}{mean}100$

[c]n=the number of control sera samples tested

Accuracy

The assay accuracy was evaluated by determining the recovery of biotin added to pooled human serum, and by estimating how well we could determine the expected amount of biotin in dilutions of a pooled

TABLE 3

Accuracy of Biotin Enzyme-linked Assay

Recovery of exogenous biotin added to a pooled human serum			Linear recovery of endogenous biotin in diluted pooled human serum[b]		
Biotin added[a] to serum (ng/L)	Amount recovered (ng/L)	Recovery (%)	Dilution	Measured values (ng/L)	Recovery (%)
120	296	114	none	560	
240	392	103	1:1	256	91
480	620	100	1:3	126	90
			1:7	82	117

[a]known concentrations of exogenous biotin were added to pooled human serum that contained 140 ng/L endogenous biotin.
[b]pooled serum sample with high endogenous biotin levels was diluted with biotin zero standard.

serum sample containing high concentrations of endogenous biotin (Table 3). The recovery of either exogenous or endogenous biotin from serum samples exceeded 89% and biotin could be measured without interference from serum components. It is therefore permissible to assay dilutions of sera with endogenous biotin concentrations that exceed the range of the standard curve, by using only zero standard as the diluent.

Validation of the assay

Figure 3 shows the results of this assay used to monitor biotin

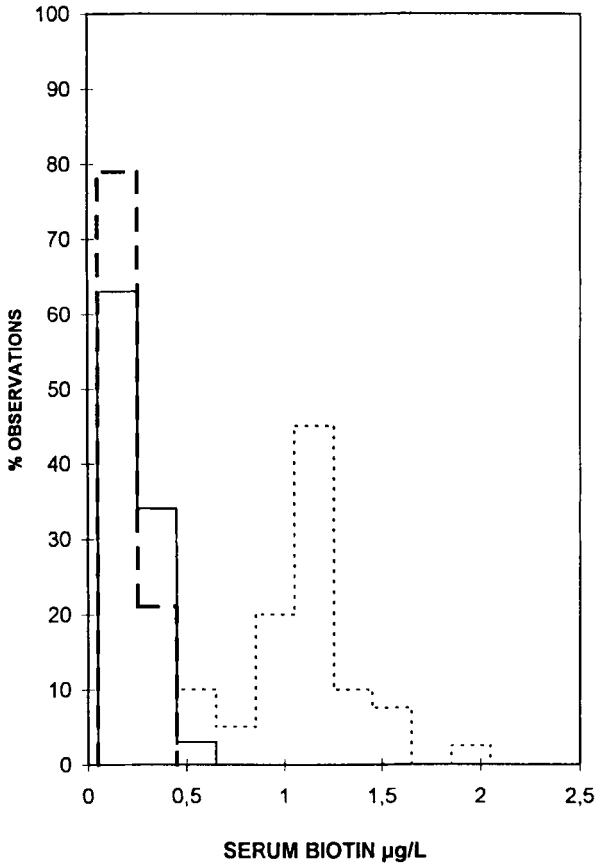

FIGURE 3. Serum biotin concentrations determined in 35 pregnant women (-- --) and in 40 patients under chronic haemodialysis treatment (- - - -) vs those determined in 68 healthy individuals (——).

concentration in three different population groups. Percentage observations were plotted against biotin concentrations in serum samples. Sixty-eight serum samples, obtained from apparently healthy adults of both sexes, were analyzed for biotin. The sera were divided into two groups of 34 and assayed in duplicate, in two different assays. The concentrations measured ranged between 66 - 600 ng/L with a mean

value of 223 ng/L. Measurement of serum samples obtained from 35 pregnant women at the final trimester of pregnancy gave rather low values for biotin, 60 - 360 ng/L, except in one of the subjects with elevated levels of biotin, i.e. in the range of those with chronic renal failure, which may be ascribable to receiving pharmacologic doses of biotin during pregnancy. Serum samples from 40 kidney-disease patients undergoing chronic haemodialysis treatment gave high values for biotin concentration, 0.56 - 1.62 μg/L. These values are in accordance with those previously reported in the literature (13) and confirm the diagnostic reliability of the method.

DISCUSSION

We describe an indirect, competitive enzyme-linked assay for measuring biotin in human serum. According to the assay method, streptavidin-HRP conjugate was preincubated with the biotin standard solutions or the unknown samples and the mixtures were then incubated with B-IgG_b immobilized on ELISA microtitre plates. The concentration of the streptavidin-HRP conjugate was such that the streptavidin binding sites were sufficient to bind apparently all the biotin present in samples, whereas, the remaining sites were inversely proportional to the amount of biotin in analysed sample. These sites could then interact with the B-IgG_b immobilized on the microtitre plates providing signal that is inversely proportional to the amount of biotin present in the assay samples. The working concentration of the streptavidin-HRP conjugate was 20 ng/ml and this was selected so as to bind approximately 50 % of the immobilized B-IgG_b in the presence of the biotin zero standard.

The preincubation step between streptavidin and biotin in assay samples was found to be necessary for measurement of endogenous biotin in human serum. This preincubation step can be omitted, with good recovery and assay linearity, when measurements are directly performed in samples containing biotin in chemically homogeneous form. Thus, the standard curves obtained following either the indirect or a direct protocol were well correlated, r = 0.997. In addition, the direct assay protocol provided good recovery of exogenous biotin added in serum samples,

ranging between 94 and 118 %. On the contrary, the linearity features of the direct assay, as determined by serially diluting serum samples containing high concentrations of endogenous biotin, were unacceptable. The above findings provide evidence that the different endogenous biotin analogues present in human serum (35), with various *Kaff* for the streptavidin-HRP conjugate each, may affect the overall analytical profile and accuracy of the assay, when a direct protocol is used.

A limited number of non-radioisotopic binding assays for determining biotin concentrations have been developed (16-27) and only few of them, to our knowledge, have been used for measuring biotin in human serum. Bayer *et al.* (16) have developed a sandwich-type competition assay using biotinylated bovine serum albumin-coated plates, streptavidin and biotinylated alkaline phosphatase; however, this assay was not evaluated in biological samples, while it requires multiple measurements of aqueous biotin solutions, at different dilution, using three overlapping standard curves with very narrow dynamic range. Chang et al. (17) have developed a competitive ELISA for determining biotin in aqueous microbiological media. Though it is sensitive, the method is rather complex and its clinical applicability has not been proved. Recently, Rosebrough *et al.* (25) developed an enzyme-linked assay based on direct competition between biotinylated peroxidase and free biotin on streptavidin-coated plates. This assay, although simple, lacks in sensitivity to detect biotin at the pmol/mL concentrations, in which this vitamin is found in normal human serum. Thus, none of the above mentioned methods, which are considered the most significant enzyme-linked binding assays reported in the literature for biotin analysis, has been evaluated for their clinical applicability to human serum samples. In addition, none of them combines low detection limit (<5ng/L), broad working range, small volume of serum needed for analysis, short assay time, and inherent simplicity for routine use in clinical laboratory.

In our laboratory, we had developed a radioligand assay (13) for determining biotin in biological fluids, based on an ^{125}I-labeled biotin derivative and avidin in solution. This assay is one of the well-studied biotin methods, which has been applied for determining biotin levels in human sera obtained from various population groups (28-30). However,

problems connected with using radiolabeled compounds such as handling, disposal, shelf life, and commercial non-availability are an obstacle to the wide application of the method and this has stimulated us to use our relative accumulated experience in developing a non-radioisotopic binding assay for determining biotin in human serum.

The assay described utilizes immobilized biotin in the form of B-IgG_b, where the remaining binding sites of the streptavidin-HRP conjugate interact. We synthesized B-IgG_b by coupling the ε-amino groups of IgG_b lysine residues to NHS-LC-biotin thus forming an amide bond. The molar ratio of ester to amino group of the carrier protein was 3:1. The conjugate preparation as described here had a consistently large yield, and after labeling, a simple dialysis of the reaction mixture was sufficient to ensure purity. The synthesis is simple, straightforward and can be done in any laboratory. We found that our ELA procedure allows the analysis of samples using a single dilution and that analysis in duplicate provides an acceptable coefficient of variation in contrast to the analysis of samples at three different dilutions (16).The method is highly reproducible and reliable as indicated by the low variations observed within and between runs, as shown in Table 1 and 2, respectively. In addition, the assay is accurate, as shown by the recovery values obtained (100-114 %) for increasing concentrations of exogenous biotin added to pooled human serum as well as by the linear recovery values obtained (90-117 %) from serially diluted (1:2 to 1:8) pooled human serum containing high endogenous biotin concentrations (Table 3). The accuracy of the assay was found to be unaffected by serum protein fluctuations as well as after the addition of BSA (up to 8 %) in pooled human serum. Serum samples were treated, before the assay, with the biotinidase inhibitor sodium *p*-hydroxymercuribenzoate, which inactivates biotinidase (31) or other enzymes containing an -SH group present in human sera that may affect assay results. The treatment is very simple and fast and does not influence the assay performance. Sera from apparently healthy individuals contained 66 to 600 ng of biotin per litre (mean 223 ng/L), while pregnant women (≥36weeks) had serum biotin concentrations (60 to 360 ng/mL), and patients undergoing chronic haemodialysis treatment showed high concentrations (0.56 to 1.62 µg/L). These values are in

agreement with those previously reported by us using the radioligand binding assay. Overall, among the advantages of the method reported, in comparison with those previously described in the literature, are: avoidance of radioisotopically labeled tracers, simple analytical protocol, short assay time, high sensitivity, broad working range of the standard curve, stability of the assay reagents for up to 12 months, and small sample volume needed for analysis. Apart from its excellent analytical characteristics, the method described is among those few which have been experimentally evaluated for their capabilitity of assessing biotin in human sera.

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