Arch. Pharm. Res. Vol.21, No.3, pp. 231-235, 1998

A Biotin-avidin Labeled Enzyme Immunoassay for the Quantitation of Serum TSH Using Protein-layered Solid Phase

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(Received September 25, 1997)

A sensitive enzyme immunoassay for serum TSH has been developed utilizing the tight binding between biotin and avidin, and three layered protein polystyrene beads as solid phase. To increase binding capacity of TSH and sensitivity of the assay, the polystyrene beads were coated sequentially with mouse immunoglobulin as first layer, rabbit antimouse immunoglobulin as second layer and monoclonal anti-TSH as third layer. A serum sample was incubated simultaneously with a monoclonal anti-TSH immobilized polystyrene beads and a second monoclonal anti-TSH covalently attached to biotin. After washing, the antibody bound serum TSH-anti-TSH-biotin complex is reacted with horseradish peroxidase (HRP)-labeled avidin. Following a second wash, the bound HRP activity was measured colorimetrically. Reproducible results were obtained within 4 hours for serum TSH in the range between 0 μIU/ml and 50 μIU/ml with detection limit of 0.1 μIU per test.

Key words : Biotin-avidin, EIA, TSH, Trilayered solid phase

INTRODUCTION

Quantitation of serum thyroid stimulating hormone (TSH) can be used in the diagnosis of primary thyroid disorders and for evaluation of secondary hypothyroidism. TSH levels are commonly measured using a radioimmunoassay. However, the normal range of TSH level is lower than 10 μIU/ml and most of the assay kit hardly manage to meet this detection range. To increase sensitivity of the assay, we introduced trilayered solid phase and biotin-avidin system into enzyme immunoassay (EIA).

Biotin, a vitamin, is a relatively small molecule (FW 244), so it can usually be conjugated to many proteins without significantly altering the biological activity of protein. A protein can be reacted with several molecules of biotin that can each bind a molecule of avidin. This greatly increases the sensitivity of many assay procedures. The avidin-biotin interaction is the strongest known non-covalent biological interaction ($Kd=10^{-15}$ M) between protein and ligand. The bond formation is very rapid and essentially non-reversible (Green, 1975). Studies with biotinyl compounds have reported that the affinity of avidin for biotin was decreased when short spacer arms were used in the biotinyl compound, apparently an effect of steric hindrance (Green *et al.*, 1971). Optimal biotin binding capabilities can be obtained by using a biotin derivative of an extended spacer arm, which reduces steric hindrance. The spacer arm also improves the complex formation of biotin with the deep biotin binding sites of avidin which is 9Å below the surface (Green *et al.*, 1971). The reduction in steric hindrance may result in an increase in sensitivity in the biotin-avidin system and its amplification system in immunoenzymatic technique were reviewed by Avrameas (1992).

In this study, we modified the solid phase coupling to anti-TSH using sequential protein layers to stretch arms to increase binding for anti-TSH in addition to the tight binding between avidin and biotin for assay. Using three protein-layered polystyrene bead as solid phase, a biotin-avidin enzyme immunoassay was developed.

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MATERIALS AND METHODS

TSH calibrators

Human TSH was obtained from Calbiochem-Behring Corporation (La Jolla, CA, USA). Calibrators were prepared by adding various concentrations of human TSH to normal rabbit serum containing 0.1% thimerosal as a preservative. TSH concentration in the cali-

brators was standardized by immunoradiometric assay (IRMA, Co-Tube™ TSH, BioRad Laboratories, Inc., CA, USA).

Solid phase-Anti-TSH

Polystyrene beads (1/4 inch diameter) were obtained from Precision Plastic Ball Co. (Chicago, IL, USA). Mouse IgG was purchased from Sigma Chemical Corp.. Rabbit anti-mouse IgG antiserum and mouse anti-TSH ascites fluid were obtained in-house. The trilayered solid phase-anti-TSH was prepared using 20 μg/ml mouse IgG in 50 mM carbonate buffer as a first layer, 1:40 diluted rabbit antimouse IgG antiserum in 100 mM PBGel, pH 8.0 as a second layer, and 1:5,000 diluted mouse anti-TSH ascites fluid (a monoclonal antibody) in 100 mM PBGel, pH 8.0 as a third layer. The solid phase-anti-TSH was stored in the third layer coupling solution with 0.01% thimerosal as a preservative. The soild phase-anti-TSH was washed three times with saline containing 0.1% Tween-20 prior to use.

Anti-TSH-biotin conjugate

Biotin-N-hydroxysuccinimide was obtained from Calbiochem-Behring Corp. (La Jolla, CA, USA). Anti-TSH IgG was purified using CNBr-Sepharose chromatography prior to biotination. Biotin was conjugated to an IgG fraction of mouse anti-TSH ascitic fluid (a second monoclonal antibody) (Savage *et al.*, 1992). The biotin-anti-TSH conjugate was prepared with buffer containing 10 mM phosphate buffer, 0.9% sodium chloride, 2% normal mouse serum, 30 mM EDTA and 0.05% Tween-20, pH 7.2. The conjugate was titrated to determine the proper concentration to be used in the biotin-avidin EIA system.

Avidin-HRP conjugate

Horseradish peroxidase and avidin were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Horseradish peroxidase was conjugated to avidin by a modification of the method of *Nakane and Kawaoi* (1974). The avidin-HRP conjugate was prepared with buffer containing 10 mM phosphate buffer, 0.9% sodium chloride, 3% normal mouse serum, 0.025% BSA, 0.025% sucrose and 0.05% Tween-20, pH 7.2. The avidin-HRP conjugate was titrated to determine the proper concentration to be used in the EIA system.

Horseradish peroxidase substrate and quencher

The substrate contains 11 mM *o*-phenylenediamine·2HCl, 16 mM citric acid, 8.9 mM sodium citrate, 2.5 mM potassium bicarbonate and 5 mM hydrogen peroxide, pH 5.0 (Gallati and Brodbeck, 1982). 0.25 M citric acid was used to quench horseradish peroxidase activity.

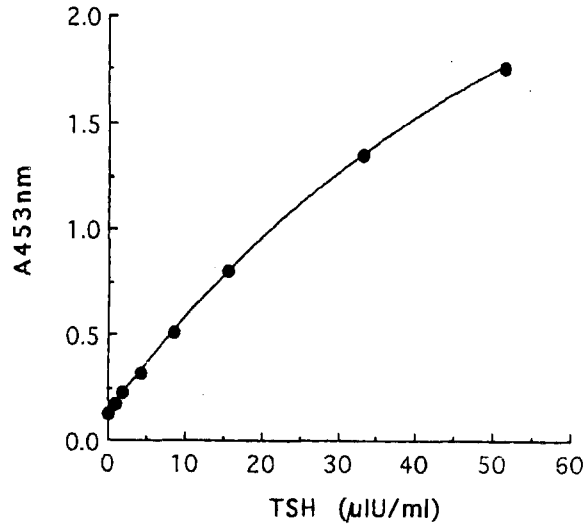

Fig. 1. Standard curve of TSH by biotin-avidin EIA. The standard curve response was obtained by biotin-avidin EIA using TSH calibrators (200 μl) and the combination of TSH monoclonal antibodies of 7D12 on trilayered solid phase (mouse IgG: 20 μg/ml as a first layer, rabbit antimouse IgG: 200 μg/ml as a second layer, and ascitic fluid of 7D12 monoclonal antibody: 1:5,000 dilution in 0.1 M PBGel as a third layer) and biotinated 5B3 (100 μl). The rest of the experiment was performed according to the biotin-avidin EIA procedure in the text.

Wash solution

0.9% sodium chloride containing 0.1% Tween-20 (V/V).

Assay procedure of biotin-avidin EIA

Pipet 200 μl of each calibrator or sample and 100 μl of the biotin-labeled anti-TSH into each well of plastic reaction tray. After adding one bead to each well, incubate for two hours in a 37°C shaker water bath. After wash and decant three times with 1 ml wash solution, add 300 μl avidin-HRP conjugate to each well and incubate for one hour at room temperature. After washing the beads, add 500 μl *o*-phenylenediamine (OPD) substrate to clean tube and then add the beads at timed intervals. After incubating for 15 minutes at room temperature, stop the reaction by adding 500 μl quencher to all tubes at timed intervals and read the absorbance at 453 nm, using water as a blank. Construct standard curve as shown (Fig. 1) and determine the TSH level from the curve.

RESULTS AND DISCUSSION

Optimization of assay component

Calibrators and assay buffer: Two sets of calibrators

were made by diluting stock TSH solution into normal rabbit serum (NRS) calibrator containing normal rabbit serum with 0.1% thimerosal and buffer calibrator containing 25 mM barbital, 100 mM NaCl, 6% BSA, 5 mM EDTA and 0.1% thimerosal, pH 8.5. Both calibrators were assayed by IRMA kit (Co-Tube™ TSH, BioRad, USA) and the following values were assigned to the calibrators (1.0, 2.0, 4.2, 8.5, 15.6, 33.2, 51.6 μIU/ml).

To compare the calibrator matrix effects, the initial correlation studies were performed using commercial human serum-based calibrators, controls and patient samples. In these experiments, we found that the EIA system can quantitate TSH in human serum-based calibrators and control serum. However, most of the patient samples gave absorbance less than zero calibrator. TSH-spiked patient samples also gave lower response than zero calibrator. To solve the problems of the lower than zero values of patient samples, TSH-spiked samples were treated three different ways and the sample recoveries were evaluated by biotin-avidin EIA. The results of three different treatment are as follows; 1) the serum dialyzed overnight produced some response over non-dialyzed sample. 2) the heat-treated serum (56°C, 1hr.) produced more response than not heated serum. 3) 30 mM EDTA addition to serum showed close response to the expected value. This results indicate that the assay buffer for the sample reaction needs to contain 30 mM EDTA and it was included in the assay buffer of anti-TSH-biotin conjugate to react with sample during the incubation period.

To verify the EDTA effects, TSH value of patient samples were evaluated by the biotin-avidin EIA procedure containing EDTA in the assay buffer using NRS calibrators and Co-Tube IRMA method. The correlation results between two methods (N=54, slope=0.973, intercept=-1.314, correlation coefficient (r)=0.977) indicate that assay buffers of anti-TSH-biotin conjugate containing EDTA is adequate using the NRS calibrator.

The evaluation was also performed for linearity using NRS calibrators with and without EDTA. TSH-spiked patient serum was diluted with NRS for the linearity study. NRS standard curve with EDTA gave percent recoveries ranging from 94% to 105%. The response was linear up to 30 μIU/ml. Samples read off the curve when NRS standard curve without EDTA was used.

Trilayered solid phase: Polystyrene bead were prewashed three times with water, followed by 50mM carbonate buffer, pH 9.5 to remove fine powder and deaerated under vacuum. The trilayered beads were prepared by coupling mouse IgG as a first layer, rabbit antimouse IgG as a second layer, mouse anti-TSH ascitic fluid as a third layer (reference solid phase). The trilayered coupling to bead was optimized in terms of protein concentration in coupling buffer, incubation time for coupling, pH and ionic strength of buffer. The evaluation of coupling efficiency was determined using gamma counter by measuring the binding ability of iodinated mouse IgG, rabbit antimouse IgG and ascites for each protein layer, with incubating 2 h at 4°C (Johnson and Thorpe, 1996). The optimum conditions for the first layer mouse IgG was 20 μg/ml in 50 mM carbonate buffer, pH 9.5, the second layer rabbit antimouse IgG was 200 μg/ml in 0.1 M PBGel (0.1 M phosphate buffer containing 0.1% gelatin), pH 8.0. Rabbit antimouse antisera 1:40 diluted solution provided similar results as 200 μg/ml of rabbit antimouse IgG. The optimum condition of ascitic fluid used in the third layer was 1:5,000 diluted solution in 0.1 M PBGel, pH 8.0. Approximately 80% of the coupling was completed within a day. Based on the EIA evaluation rabbit antimouse antiserum in second layer showed better response than purified IgG.

As mentioned previously for the phenomenon that the lower absorbance reading than zero calibrator in patient serum sample, it was proposed that complement present in patient sample may bind the Fc portion of rabbit antimouse IgG in the second layer, and it could show inhibitory binding of anti-TSH to rabbit mouse IgG in the second layer which, subsequently reduce TSH binding in sample. The preliminary studies using dialyzed serum, heat treated serum (1 hr. 56°C), and EDTA-spiked serum supported that the complement interferes in recovery of TSH value. For further test of the complement hypothesis, rabbit antimouse $F(ab')_2$ and protein A were used for the second layer coupling. Thus, three sets of trilayered solid phase were prepared as follows and evaluated by TSH-spiked patient samples ranging from 1 to 50 μIU/ml to find out the recovery improvement. 1) reference beads which coupled mouse IgG as a first layer, rabbit antimouse IgG as a second layer, mouse anti-TSH ascitic fluid as a third layer. 2) $F(ab')_2$ beads which coupled mouse IgG as a first layer, rabbit antimouse $F(ab')_2$ as a second layer, mouse anti-TSH ascitic fluid as a third layer. 3) Protein A beads which coupled mouse IgG as a first layer, protein A as a second layer, mouse anti-TSH ascitic fluid as a third layer. The initial study indicated that $F(ab')_2$ beads can quantitate TSH in the patient samples using NRS calibrators without EDTA. The reference beads also can quantitate TSH in the patient samples using NRS calibrators with EDTA. However, protein A beads gave lower response and not quantitate TSH values properly. The results indicate that EDTA in assay system could prevent complement effect and provide similar result as $F(ab')_2$ in the second layer which considered not to provide complement binding effect.

HRP enzyme substrate and quencher: The chromogens of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)·2 ammonium salt (ABTS) and hydroxydichloro-

benzene sulfonate were compared to OPD in EIA assay system. The OPD substrate gave a better response than either of the other two. The antioxidants, sodium bisulfite and ascorbic acid, were evaluated for their ability to inhibit endogenous color development in OPD substrate reagent. Both compounds had an inhibitory effect on the peroxidase enzymatic activity and were avoided to use. As enzyme quenchers, 0.4 M sodium azide and 0.25 M citric acid were evaluated and optimum reading wavelength were 430 nm and 453 nm, respectively. We found both performed well.

Assay buffer: The optimum concentrations of NRS, Tween-20, EDTA and other additives were studied for anti-TSH-biotin and avidin-HRP conjugates. The optimum concentration was 2% normal mouse serum, 0.05% Tween-20 and 30 mM EDTA in 10 mM PBS, pH 7.2 for biotin conjugate, and 0.025% BSA, 0.025% sucrose, 3% normal mouse serum, 0.05% Tween-20 in 10 mM PBS, pH 7.2 for avidin-HRP conjugate.

Wash buffer: Wash buffer was studied for saline and PBS, with and without Tween-20. There was no significant difference between saline and PBS, although Tween-20 is definitely required.

Assay development

Screening of compatible monoclonal antibody clones: The monoclonal antibody clones, 1A12, 5B3, 2C10, 7D12 (Biostride, CA, USA) were studied by preparing solid phase coupling and 7D12, 5B3 for biotin conjugate. The result of screening study indicates that the combination of 5B3-biotin with 7D12-solid phase are the best among tested (Table I).

Non-specific binding: High non-specific binding of avidin-HRP to solid phase in the absence of biotin conjugate when assayed with horse serum calibrators, was not observed with rabbit serum calibrators. This result indicates that calibrator matrix of NRS is better than the matrix of horse serum.

Titration of 5B3-biotin: 5B3-biotin was titrated and the optimal concentration was 0.11 μg/assay. However, increasing concentration of 5B3-biotin from 0.55 μg/assay to 2.75 μg/assay resulted in higher non-specific binding without gaining any specific binding.

Table I. Screening of compatible monoclonal antibody clones for solid phase coupling and biotination.

Biotin conjugation / Solid phase coupling		Absorbance at 453 nm					
		7D12		5B3			
		1A12	5B3	2C10	1A12	2C10	7D12
Calibrators (μIU/ml)	0	0.136	0.822	0.257	0.169	0.164	0.161
	2.9	0.130	1.123	0.266	0.192	0.179	0.226
	35	0.158	1.159	0.258	0.176	0.427	0.429

Performance evaluation

The sensitivity was determined by calculating the mean of two standard deviations at zero point using 20 replicates of zero calibrator. The smallest quantity of TSH which could be consistently distinguished from background was 0.1 μIU/test. Serum samples were randomly selected to be in the range of zero to 50 μIU/ml to evaluate correlation with the other method. The serum samples were assayed by a IRMA kit (BioRad Labs., CA). There was a good agreement between TSH values obtained by biotin-avidin EIA method and IRMA method. Correlation coefficient (r) was 0.98. TSH values lower than or equal to 10 μIU/ml also showed good correlation between biotin-avidin EIA and IRMA method (Table II). The above results indicate that the biotin-avidin EIA method using trilayered solid phase showed similar sensitivity to that of IRMA or ELISA using alkaline phosphatase polymers and bispecific F $(ab')_2$ fragment against TSH and alkaline phosphatase (Morimoto and Inouye, 1997) which claimed 30 times better sensitivity than that of conventional EIA using enzyme-monoclonal antibody conjugate.

Serial dilution of a patient sample with high TSH concentration gave linear response. Study with patient samples spiked with known quantities of TSH gave recoveries of 105% to 107% with a mean of 106% (Table III). Assay precisions were studied at two concentrations of controls for every assay (Table IV). Within-run coefficient of variations (n=10) were between 6.7% and 4.9% for control samples containing TSH at 16 and 37 μIU/ml. Coefficient of variations of the run-to-run assay (n=9) ranged between 8.1% and 6.3% for the same controls in the within-run assay.

In the biotin-avidin EIA method described, TSH in the patient sample is reacted simultaneously with a monoclonal anti-TSH immobilized on a protein layered polystyrene bead and a second monoclonal anti-

Table II. Correlation of TSH values by biotin-avidin EIA and IRMA

TSH (μIU/ml)	x	y	n	γ	Slope	Intercept
0~50	IRMA	B-Av EIA	61	0.984	0.998	-0.416
0~10	IRMA	B-Av EIA	37	0.936	0.978	-0.035

B-Av EIA (biotin-avidin EIA)
IRMA (immunoradiometric assay, Co-Tube™ TSH)

Table III. Recovery study of TSH spiked serum

TSH added (μIU/ml)	Serum TSH in patient sample (μIU/ml)	Calculated total TSH (μIU/ml)	TSH recovery	
			(μIU/ml)	%
18.3	6.1	24.4	25.6	104.9
18.3	2.8	21.1	22.4	106.2
2.8	6.1	8.9	9.5	106.7

Table IV. Assay precision study

	Number of assays	Mean (μIU/ml)	Standard deviation (μIU/ml)	Coefficient of variation (%)
Inter-assay				
Serum A	10	37	2.47	6.7
Serum B	10	16.3	0.79	4.9
Intra-assay				
Serum A	9	35.5	2.86	8.1
Serum B	9	12.0	0.75	6.3

TSH covalently attached to biotin. After washing, the resultant complex (solid phase-αTSH-TSH-αTSH-B) is reacted with HRP-labeled avidin. Following a second wash, the HRP activity present in the final complex (solid phase-αTSH-TSH-αTSH-B-AV-HRP) is measured colorimetrically. The color intensity is directly proportional to TSH concentration in the sample. This biotin-avidin EIA is reproducible and relatively rapid, requiring only four hours to perform. The assay can measure as little as 0.1 μIU/test in serum samples. The performance characteristics of the biotin-avidin EIA were sensitive as well as IRMA and acceptable to quantitate low concentrations of TSH (lower than 10 μIU/ml) in the sample using non-isotopic label and ordinary laboratory equipment.

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