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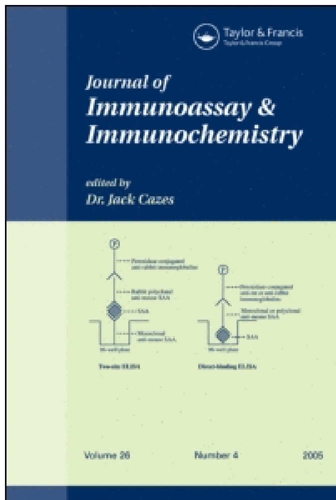

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An Enzymeimmunoassay for Total Thyroxine Using Avidin-Biotin Separation System and Thyroxine-Peroxidase Conjugate

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AN ENZYMEIMMUNOASSAY FOR TOTAL THYROXINE USING AVIDIN-BIOTIN SEPARATION SYSTEM AND THYROXINE-PEROXIDASE CONJUGATE

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ABSTRACT

An avidin-biotin enzymeimmunoassay for total thyroxine in serum is described. Avidin was adsorbed to biotinylated bovine serum albumin coated tubes prepared with glutaraldehyde as coupling agent. In the enzymeimmunoassay, affinity purified biotinylated anti-thyroxine IgG, sample or standards, and thyroxine-horseradish peroxidase were simultaneously added to the avidin coated tubes. The bound enzymatic activity was then measured with o-phenylenediamine and H_2O_2. Results showed that the assay has good precision (within-assay CV% less than 10% and between assay 11.7% in hypo- and 6.9% in hyperthyroid range), good assay range (0-800 nmol/L), good sensitivity (4 nmol/L), and can be performed in 2.5 hours. The results obtained correlate well (r=0.93) with those of an RIA.

(KEY WORDS: Enzymeimmunoassay, avidin-biotin, thyroxine.)

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INTRODUCTION

Measurement of thyroxine (T_4) in serum has been long recognized as providing a valuable aid in the clinical diagnosis of various thyroid disorders. The non-isotopic techniques for T_4 determination divide into 2 main groups. The first includes homogenous immunoassays based on fluorescence polarization (1),

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fluorescence transfer (2), or enzyme inhibition (3). These methods which do not necessitate the separation of the bound and the free fractions may be fully automated but do require sophisticated equipment. The second group includes heterogeneous immunoassays generally using a T_4-enzyme conjugate and can be carried out with currently available equipment. In most cases, the enzymatic conjugate has been prepared by chemically coupling T_4 to horseradish peroxidase (HRP) (4), alkaline phosphatase or beta-galactosidase (5) and bound/free separation has been achieved with anti-T_4 antibodies immobilized on a plastic support (6) or using the double antibody technique (4).

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We have developed a novel enzymeimmunoassay (EIA) method for total T_4 determination which contains biotinylated sheep anti-T_4 I_gG, avidin immobilized tubes and T_4-HRP conjugate. The avidin/biotin system was incorporated into the EIA to increase sensitivity because of high affinity of avidin for biotin (affinity constant = 10^{15}M). In our competitive T_4-EIA system, sample or standards, T_4-HRP conjugate, and biotinylated anti-T_4 IgG were simultaneously added, to the avidin coated tubes in the presence of a blocking agent 8-anilino naphthalene-1-sulfonic acid ammonium salt. After 2 h incubation at 37°C, the tubes were washed and enzymatic activity bound to solid phase was measured.

MATERIAL AND METHODS

Avidin, N-hydroxy succinimido biotin (BNHS), L-T_4 (free acid), o-

phenylenediamine (OPD), glutaraldehyde, thimerosal sodium salt, horseradish peroxidase (HRP), bovine serum albumin (BSA), the blocking agent 8-anilino naphthalene-1-sulfonic acid ammonium salt (ANS), tween 20, dimethyl formamide (DMF), H_2O_2 and 2,4,6-trinitrobenzene (TNBS) were obtained from Sigma (St. Louis, MO, USA). Sephadex G-25 and sephacryl S-200, sepharose CL-4B were purchased from Pharmacia, Uppsala (Sweden). Sheep anti-T_4 IgG was from ILS (International Laboratory Service, U.K.).

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Preparation of Immunoglobulin Fraction

20 ml of sheep anti-T_4 antiserum was fractioned with Na_2SO_4 according as previously described (7). The Na_2SO_4 concentration used to precipitate the IgG fraction was 18%.

Preparation of Thyroxine-substituted Sepharose-4B

Thyroxine was coupled to CNBr-activated sepharose-4B following the method recommended by Pensky et al (8). After consecutive washing of CNBr-activated sepharose-4B with HCl and $NaHCO_3$, L-thyroxine, dissolved in KOH, was added to the stirred suspension of gel in the ratio of 2 mg T_4 for each ml of the original settled sepharose-4B. The final product was stored at 4^oC in 0.1 M $NaHCO_3$ buffer.

Affinity Purification of Sheep Anti-T_4

Immunoadsorption of 15 ml sheep anti-T_4 IgG (10 mg) on 3.5 ml settled T_4-coated solid-phase (CNBr-activated sepharose-4B) was carried out by continuous mixing on an end-over-end mixer for 2 days at 4^oC. The gel was then packed into a small column and washed with 0.1 M $NaHCO_3$. The selective elution of the immunoadsorbed sheep anti-T_4 IgG was carried out by stepwise reduction of pH (pH 6, pH 5, pH 4, pH 3.5, pH 3).

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Biotinylation of Affinity Purified Sheep Anti-T4 IgG

Biotinylation was carried out according to the procedure described by Guesdon et al (9). The ratio of added BNHS (mg) to the IgG solution (ml) for biotinylation was between 1 and 1.6

Determination of Free Amino Groups in Biotin-Substituted and in Native Affinity Purified Sheep Anti-T4 IgG

The free amino groups in the IgGs were determined following the method developed by Synder et al (10). 25 ul of 7.5 mM TNBS in 0.1 M borate buffer pH 9.3 was added to 1 ml of sample diluted in the same buffer and incubated 30 min at room temperature. The optimum TNBS concentration was determined by using glycyl glycin as substrate. The absorbances were read at 420 nm. The reagent blank consisted of 25 ul of 7.5 mM TNBS in 1 ml of 0.1 M borate.

Conjugation of T4 to HRP

T_4 was conjugated to HRP by the two step glutaraldehyde method as described by Avrameas (11). Ten mg of HRP was activated with 1% glutaraldehyde and concentrated to 1 ml. Ten mg of T_4 dissolved in NaOH/DMF mixture were added dropwise with stirring. After overnight incubation the unconjugated residual T_4 was eliminated by sephadex G-25 filtration. The purification of T_4-HRP conjugate was done by sephacryl G-200 chromatography. The elution buffer was 50 mM tris pH 8.2. The T_4-HRP conjugate containing peak was stabilized with 1% BSA and 0.01% thiomerosal mixed with an equal volume of glycerol and stored aliquoted at -20°C.

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Preparation of Avidin-Coated Tubes

After the tubes were washed with distilled water, 300 ul of phosphate buffer saline (PBS) containing 1 g of BSA per liter was added and the mixture was gently mixed for 2h. The tubes were rinsed with distilled water to remove fines and were allowed to stand for 16 h with 300 ul of 10 ml/L glutaraldehyde solution. They were then rinsed 10 times with distilled water to remove any unbound material, and then incubated for 6 h with 300 ul of the solution of 30 mg/L biotinylated BSA in PBS (9 ug/tube). The biotinylation of BSA was performed as described for biotinylation of antibody (see above). After rinsing with distilled water, 300 ul solution of PBS containing 1 g of BSA and 30 mg avidin per liter was added to each tube and

incubated for 16 h. After rinsing with distilled water, the tubes were coated by incubating for 20 min with PBS containing 1 g of BSA and 25 g of sucrose per liter, and then allowed to dry thoroughly. When dry, they were placed in plastic bags, sealed tightly, and stored at -70°C.

T4 Standards

T_4-free serum was prepared by removing T_4 from pooled human serum by charcoal treatment. 1 mg of L-T_4 (free acid) was suspended in water, dissolved by adding 20 ul of 1 mol/L NaOH solution and then calibrated against calibration material from the DPC (USA) test kit and diluted in the T_4-free serum to give a stock solution of 200 mg/L. We added known amounts of this stock solution of T_4 to give final concentrations of 0, 3.1, 6.2, 12.5, 25, 50, 100, 200, 600, 1200 ug/L in stripped human serum.

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Substrate Solution

The substrate solution was freshly prepared in 0.1M citrate buffer pH 5.5, H_2O_2 0.02% OPD 2 g/L (final pH 5.0) and protected from light.

EIA Procedure

Avidin coated tubes were washed 3 times with PBS containing 1 g/L albumin and inverted for 5 min on adsorbent paper. Duplicate 100 ul aliquots of T_4 standards

or unknown were delivered into the tubes, followed immediately by additon of 100 ul with 0.5 ml of 4N H_2SO_4. Absorbances were read at 492 nm and plotted against T_4 concentrations.

RESULTS

Affinity Purification of Anti-T4 IgG

Sheep anti-T_4 IgG eluted predominantly at pH 6.5 (fractions 34-39) with lesser amounts at pH 5 (fractions 46-48), pH 4 (fractions 56-58) and pH 3.5 (fractions 63-64). The dilution of each pool which bound 50% of T_4-HRP was used for the preparation of EIA standard curves in order to estimate their respective affinity constants by Scatchard analysis. Immunoglobulins with the highest affinity ($K=4x10^{13}$ L/Mol) were eluted at pH 3.5 while IgG which eluted at pH 5 and pH 4 represented populations of antibodies with lower affinities. The IgGs with the second highest affinity ($K=1.5x10^{11}$) were eluted at pH 6.5. The pH 3.5 pool was used for development of the T_4 EIA.

Biotinylation of Affinity Purified Sheep Anti-T4 IgG

Due to its greater solubility in aqueous solutions, Biotinyl-N-hydroxysuccinimide (BNHS) was used. The degree of substitution was determined by TNBS. The optimum concentration and final dilution of TNBS were chosen with minor modifications on the basis of the study of Synder and Sabocinski (10).

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To optimize biotinylation we tested several different conditions and concentrations of biotin. In order to obtain the desired molar ratio of BNHS to free amino groups of the sheep anti-T_4 IgG, a solution of 0.1M $NaHCO_3$ containing 1 mg of protein was mixed with various volumes of 0.1M solution of BNHS in distilled DMF (34.1 mg/dl). For this, 15, 35, and 70 ul of 0.1M BNHS corresponding to 0.5, 1.6, 3.26 mg of BNHS respectively were added to antibody solution. The degree of biotinylation was determined by measuring the number of free amino groups on the anti-T_4 IgG before and after biotinylation with TNBS. It can be seen from Figure 1 that the best result was obtained when a concentration of 1.6 mg BNHS was employed. Above this concentration, an increase in the degree of biotinylation and below this concentration, decreasing substitution of amino groups by biotin was observed. At the same time, the effect of biotinylation with three different concentrations of BNHS (0.5, 1.6, 3.26 mg) on the immunoreactivity of sheep anti-T_4 IgG was determined using the dilution curves prepared with native and biotin substituted sheep anti-T_4 IgGs. The dilution curves shown in Figure 2 indicate that the antigen binding capacity of sheep anti-T_4 IgG decreased with increasing biotin substitution. When biotin-labelled anti-T_4 IgG was prepared with 0.5 mg of BNHS, the antigen binding capacity remained unchanged. Compared to native antibody, the derivatives prepared with 1.6 and 3.26 mg of BNHS showed a decrease in antigen binding capacity. The decrease in the antigen binding capacity of antibodies biotinylated with high concentration of BNHS has been previously reported (15,16).

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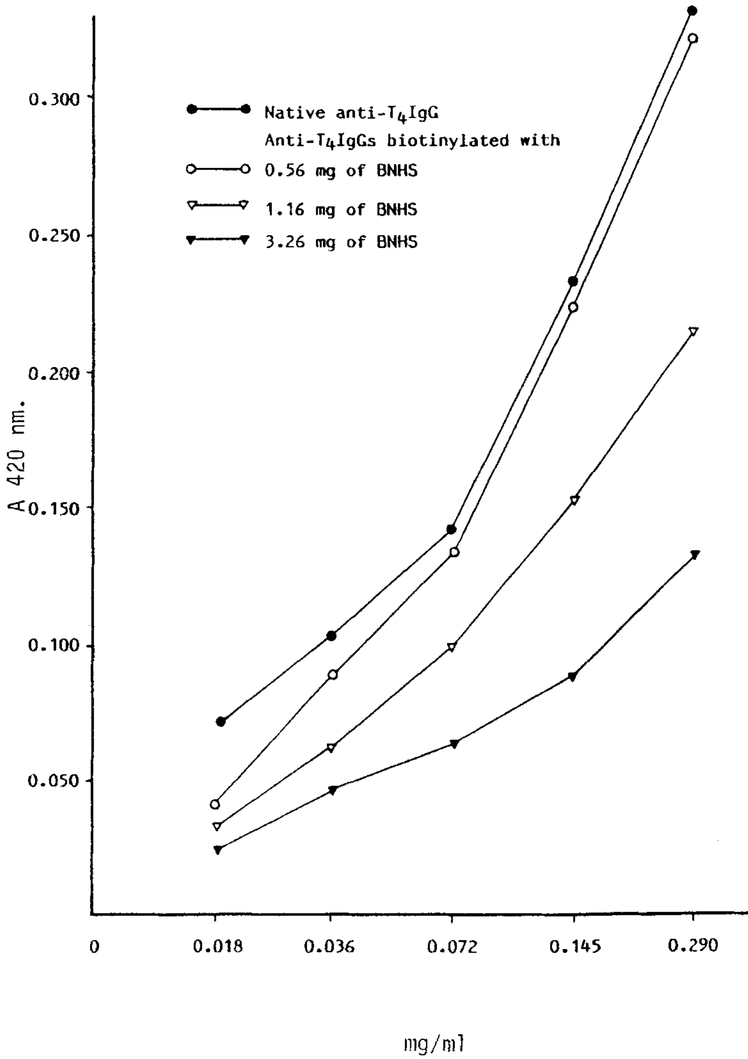

Figure 1: Comparison of the reaction of TNBS with sheep anti-T_4 IgG and sheep anti-T_4 IgGs biotinylated with three different concentrations of BNHS (0.5, 1.6, 3.26 mg).

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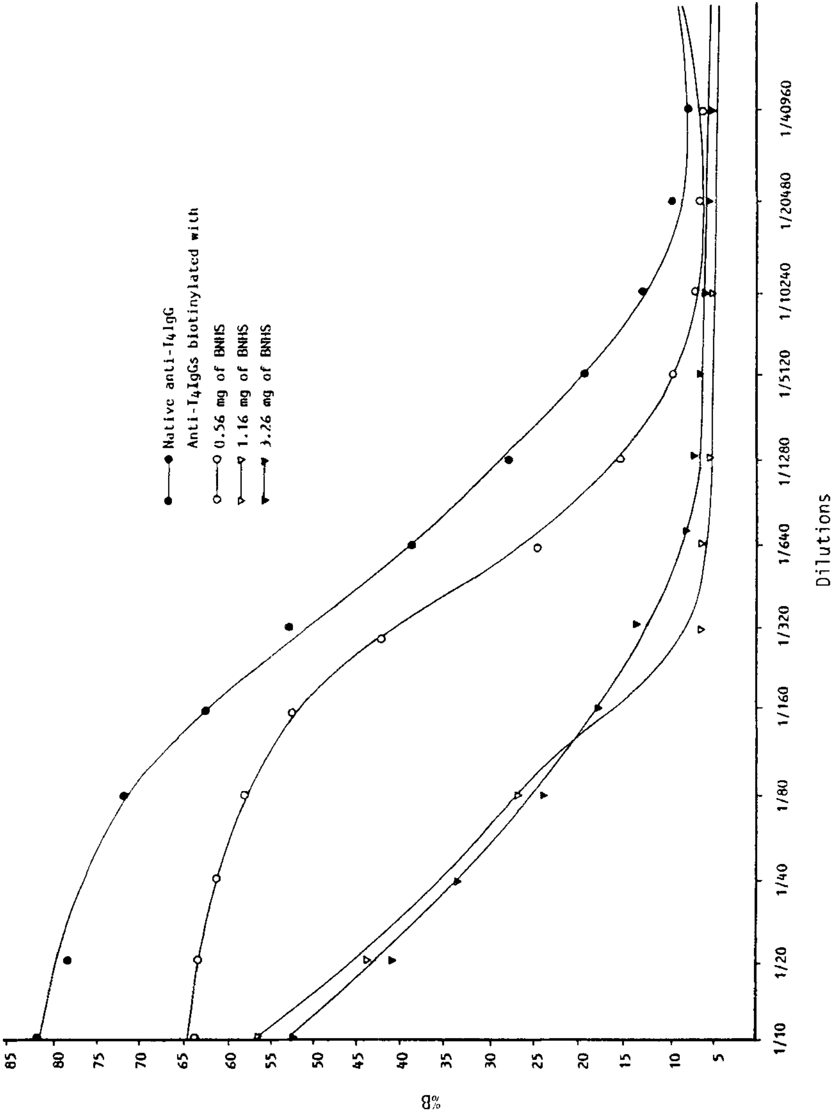

Figure 2: Comparison of dilution curves of sheep anti-T_4 IgG before and after biotin substitution.

Since the antigen binding capacity of biotin substituted antibody was affected by high degree of biotinylation, antibody biotinylated with 1.6 mg of BNHS was used in T_4-EIA.

Optimization of Avidin Coating

In initial studies, an avidin containing probe (avidin-HRP) was employed in the two different systems. Both of them contained biotinylated anti-T_4 IgG. One of these methods used anti-T_4 IgG coated tubes, and the other BSA-T_4 coated tubes. Both systems resulted in high non-specific binding produced by binding of avidin-HRP conjugate to solid-phase. This high non-specific binding observed with avidin-HRP conjugate systems was eliminated here by the use of tubes coated with avidin alone.

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In initial studies of the coating procedure, the effect of glutaraldehyde treatment of assay tubes (1% v/v) on the adsorption of biotinylated BSA and the optimum concentration of biotinylated BSA for coating were tested. The concentrations of biotinylated BSA tested for coating in PBS ranged from 5 to 50 mg/L. After adsorption of biotinylated BSA to the tubes and thorough washing, all tubes were treated with constant amount of avidin (50 mg/L) at room temperature overnight, to determine optimum concentration of biotinylated BSA. After further washing, binding of avidin was assessed by adding excess biotinylated anti-T_4 IgG (100 ul, 0.07 ug/tube) and 100 ul of 1/100,000 dilution of T_4-HRP. Incubation for 2

h at 37°C, followed by washing, and addition of substrate. The maximum adsorptivity of biotinylated BSA was at 30 mg/L. The glutaraldehyde treatment of the tubes markedly enhanced the adsorption of biotinylated BSA comparing to a simple buffer coating of biotinylated BSA in PBS. The optimum biotinylated BSA concentration (30 mg/L) was used for all further studies.

To determine optimum avidin concentration for the coating, serial concentrations of avidin were prepared ranging from 0 to 100 mg/L and 25 biotinylated BSA coated tubes were studied for each concentration of avidin. After incubation of biotinylated BSA coated tubes with varied concentrations of avidin and washing, binding of avidin was assessed by addition to the tubes of 100 ul of 1/100,000 dilution of T_4-HRP and excess biotinylated anti-T_4 IgG (100 ul, 0.07 ug/tube), using a concentration sufficient to bind 50% of conjugate. The effect of changing avidin concentrations of the coating solution on the capacity of the coated tubes to bind a fixed amount of T_4-HRP conjugate is shown in Figure 3. There was a progressive increase in binding of T_4-HRP as the concentration of avidin was increased, reaching a plateau at 30 mg/L. This concentration of avidin was used for all further coating procedures. The sensitivity of the present method was better than that obtained with biotinylated anti-T_4 IgG, and BSA-T_4 coated tubes.

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Performance Characteristics of EIA

A representative standard curve over the range 0-800 nmol/L of T_4 is shown in Figure 3. The detection limit as calculated from the standard deviation of

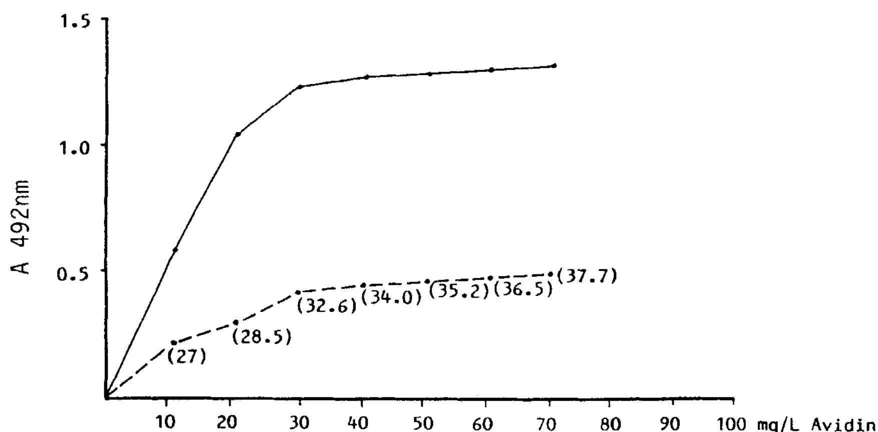

Figure 3: Relationship between avidin concentrations used in the coating procedure and the capacity of the resulting solid phases to bind a fixed amount of T_4-HRP conjugate in the presence of the zero standard (full line) or the 400 nmol/L T_4 standard (dotted line). The numbers in brackets indicate percentage of the ratios: absorbance of 400 nmol/L standard/absorbance of zero standard.

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absorbances for 20 replicates of the zero concentration standard at the 95% confidence level, was 4 nmol/L.

The assay precision was carefully assessed by testing 3 serum samples from hypo-, eu-, and hyperthyroid patients, 10 times each on at least 20 occasions, including different batches of avidin coated tubes. The results are shown in Table 1. The specificity of the T_4 enzymeimmunoassay was verified by testing various chemically related compounds or known metabolites. Negligible cross-reactivity was observed with MIT, DIT and T3 in contrast to high cross-reactivity obtained with D-thyroxine. Serial dilutions of a high T_4 serum in the zero T_4 standard were assayed for their T_4 content. The measured concentration varied linearly with dilutions. In a

TABLE 1

Precision of the T_4 EIA

	Patient Groups		
	Hypothyroid	**Euthyroid**	**Hyperthyroid**
Within-run precision (average of 20 CV%)	8.9	5.2	6.5
Between-run precision Number of assays	20	20	20
Mean nmol/L	9	17	300
CV%	11.7	9	6.9

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blind study, 40 serum samples originating from patients with different thyroid states were assayed both by EIA and RIA (DPC, USA) methods. Excellent agreement was observed with RIA; T_4 (RIA, DPC) = 0.89 T_4 EIA + 6.85, r = 0.93.

DISCUSSION

We describe use of avidin coated tubes, to isolate T_4-HRP conjugate bound to affinity purified sheep anti-T_4 IgG labelled with biotin. In the development process we encountered high nonspecific binding, especially in a system in which anti-T_4 IgG coated tubes were used to isolate biotinylated anti-T_4 IgG and avidin-HRP conjugate. Similarly, an assay in which BSA-T_4 coated tubes, biotinylated anti-T_4 IgG and avidin-HRP conjugate were employed yielded a high background absorbance. High nonspecific binding using an avidin-containing probe has been described by others (12-14). This high nonspecific binding was eliminated in our technique by the use of avidin coated tubes.

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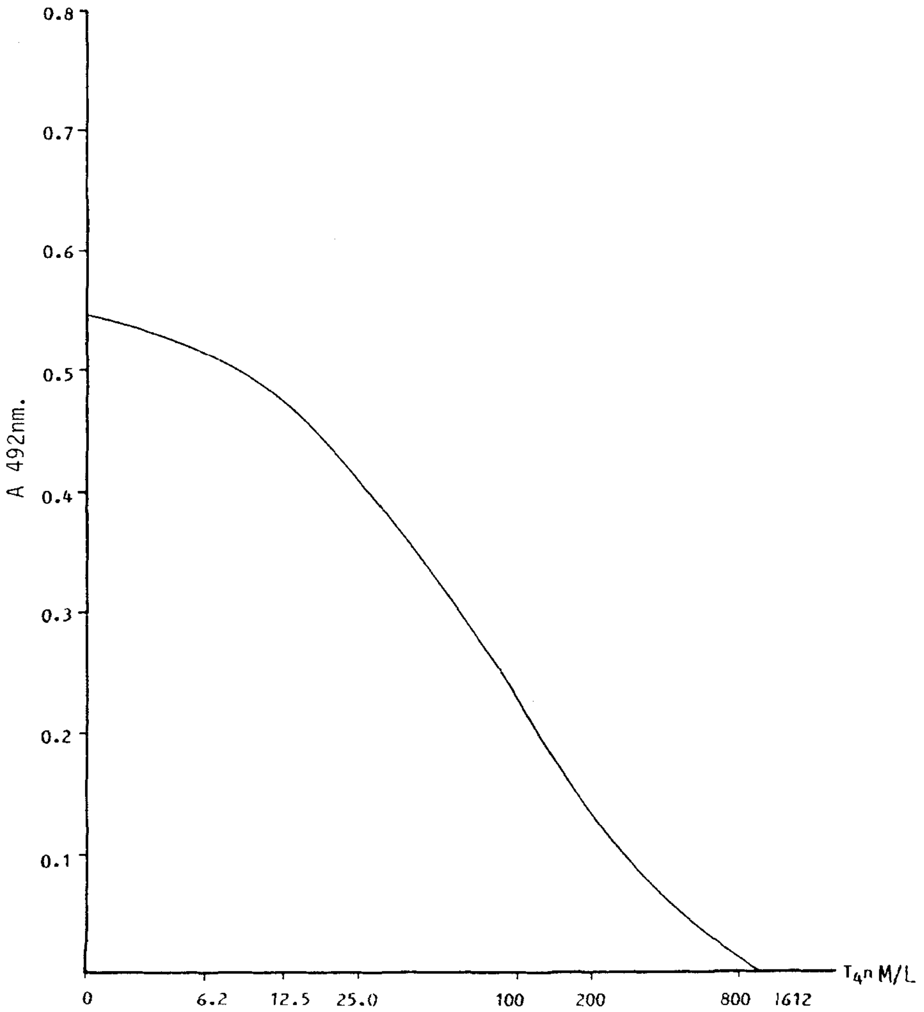

Figure 4: Representative standard curve for the T_4 enzymeimmunoassay.

In this method, affinity purified sheep anti-T_4 IgG was used to improve specificity. This simple and convenient T_4-EIA has performance characteristics which are as good as those of the RIA for T_4 developed by a commercial company (DPC, USA). The present EIA has acceptable reproducibility (Table 1), sensitivity (4 nmol/L from standard curve in Figure 4) and good correlation with the RIA. The assay specificity assessed on the basis of cross-reaction studies and a parallelism experiment was excellent. The enzymeimmunoassay presented here permits specific and accurate determination of total T_4 using standard laboratory equipment.

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