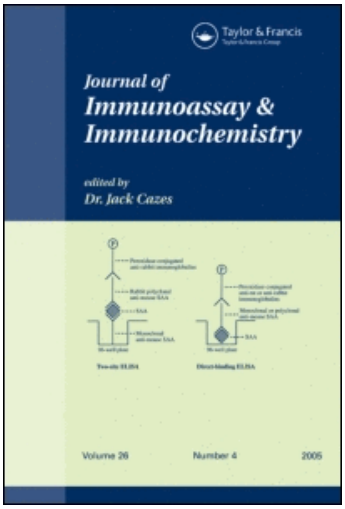

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 04 March 2002

To cite this Article Zacharopoulou, Anastasia D. , Christofidis, Ion , Kakabakos, Sotiris E. and Koupparis, Michael A.(2002) 'FREE THYROXINE SOLID-ANALOG IMMUNOASSAYS. INVESTIGATION OF THE ALBUMIN EFFECT ON THE ANTIBODY BINDING TO IMMOBILIZED THYROXINE-PROTEIN CONJUGATES', Journal of Immunoassay and Immunochemistry, 23: 1, 95 — 105
To link to this Article: DOI: 10.1081/IAS-120002277
URL: http://dx.doi.org/10.1081/IAS-120002277

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 23(1), 95–105 (2002)

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ABSTRACT

A reliable one-step, analog-based FT4 immunoassay should be characterized by minimal binding of the serum thyroxine-binding proteins to thyroxine-analogs, used either as liquid-phase tracers or as solid-phase reagents. In this work, we investigated the effect of serum albumin concentration on the anti-T4 antibody binding to immobilized T4-protein conjugates with respect to the molecular weight (MW) and the T4-to-protein molar ratio of the conjugates. It was found that the presence of albumin in the serum sample, at

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concentrations up to 120 g/L, slightly decreased the antibody binding to the immobilized conjugates (less than 10%). In addition, this decrease was independent of both the MW and the T4-to-protein molar ratio of the conjugates. On the other hand, using the same conjugates as liquid-phase labeled analogs, the observed decrease ranged between 2 and 33%, depending on the MW of the conjugate. These findings indicate that the use of immobilized T4-protein conjugates may improve FT4 immunoassay reliability, concerning, especially, the issues of serum albumin concentration and/or affinity alterations.

INTRODUCTION

During last years, one-step competitive analog-based immunoassays for free thyroxine (FT4) have found widespread application. This is due to the fact that these methods are simple, rapid, low-cost, and can be easily automated.(1) Nevertheless, the reliability of these methods was questioned when they were first introduced.(2,3) The guidelines addressed by the American Thyroid Association, concerning the development of a reliable analog-based immunoassay for FT4, have emphasized the requirement for minimal binding between the T4-analogs and the serum T4-binding proteins. (4) This binding could affect the determination of FT4 in certain pathological situations (e.g., non-thyroidal illness), which are characterized by altered concentration and/or affinity of the serum T4-binding proteins, and especially of albumin.(1,3,5) As it is reported in the literature, the reliability of the current analog-based FT4 immunoassays has been improved, as compared to the former ones.(1,6,7) This improvement has been ascribed, at least in part, to the use of T4-analogs as solid-phase reagents instead of liquid-phase tracers.(8) It has been suggested that the attachment of the analog to solid support creates a ''macroanalog,'' which may have substantially different properties from the same analog in liquid phase and, thus, binds less to serum albumin.(1,8) However, to our knowledge, no experimental evidence supporting this assumption has been reported in the literature.

Here, we present the results of our attempt to clarify this matter. For this purpose, we investigated the effect of serum albumin concentration on the binding of anti-T4 antibody to immobilized T4-protein conjugates with respect to the molecular weight (MW) and the T4-to-protein molar ratio of the conjugates. In order to obtain solid conclusions concerning the properties of the immobilized conjugates, as compared to the properties of the

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liquid-phase ones, the T4-protein conjugates prepared were used either as solid-phase reagents or as liquid-phase labeled analogs.

EXPERIMENTAL

Materials

Levo-thyroxine (T4) sodium salt pentahydrate, rabbit gamma-globulins, horse spleen ferritin, bovine thyroglobulin, bovine serum albumin (BSA), porcine gelatin, goat anti-mouse IgG antibody–horseradish peroxidase conjugate (2nd Ab-HRP), goat anti-mouse antiserum, mouse gamma-globulins, gel filtration molecular weight markers, and BCG albumin reagent were obtained from Sigma Chemical Co (St. Louis, MO, USA). Human transferrin was from Fluka (Germany). *p*-Benzoquinone (PBQ) was obtained from Ferac (Germany). 2,2′-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets were supplied by Boehringer Manheim GmbH (Germany) and were used for the preparation of the enzyme-substrate solution according to the instructions of the manufacturer. The monoclonal anti-T4 antibody (MAb anti-T4) was from OEM Concepts Inc. (USA). Thyroid hormone stripped human serum albumin (HSA) was obtained from Scipac (UK). The microtitration wells (F8 Maxisorp Loose) were from Nunc A/S (Denmark). Sephadex G-25 and Sephacryl S-300 were from Pharmacia (Sweden). Radioiodinated T4 (^{125}I-T4, specific radioactivity 300 μCi/μg) was a product of I./R.-R.P., N.C.S.R. ''Demokritos'' (Greece). The zero standard used was T4, T3, and TSH stripped human serum (Scantibodies Laboratories, USA). All the other chemicals and reagents were from Merck (Germany), unless otherwise indicated. The water used throughout this study was doubly distilled.

Preparation of T4-Protein Conjugates

T4 was conjugated to the different proteins following a slightly modified, previously described methodology.(9) Briefly, the proteins were dissolved at a concentration of 5.0 g/L in 0.33 M phosphate buffer, pH 6.0, containing 0.1 M NaCl. Appropriate volumes of freshly prepared PBQ solution, 30 g/L in ethanol, were added to each one of the protein solutions in order to ensure a 10-fold excess of PBQ per amino group of protein. The mixtures were incubated for 1 h at room temperature (RT) in the dark. The activated proteins were separated from the unbound PBQ by size exclusion chromatography on a Sephadex G-25 column (1 × 30 cm),

which was equilibrated and eluted with 0.15 M NaCl solution. The activated protein was collected in one fraction (1.5 mL). In this fraction 150 μL of 0.1 M carbonate buffer, pH 9.2, 3.0 μL of radioiodinated T4 (^{125}I-T4, 400 μCi/mL), and 15 μL of 10 g/L T4 solution in dimethylsulfoxide were added.

The mixtures were incubated overnight at RT. Afterwards, 0.165 mL of 1 M lysine solution, pH 7.0, were added in the reaction mixture and were incubated for 2 h at RT. The T4-protein conjugate was separated from the unbound T4 by passing the reaction mixture through a Sephadex G-25 column (1 × 30 cm), which was equilibrated and eluted with 0.05 M carbonate buffer, pH 9.2, containing 0.5 g/L NaN_3. After elution of the conjugates, the unbound T4 was eluted with the same buffer containing 10 g/L BSA. The presence of protein in the fractions was determined by reading the absorbance at 280 nm. The presence of T4 in the fractions was detected by measuring the radioactivity. Fractions having high optical absorbance at 280 nm and high radioactivity values, corresponding to the T4-protein conjugate, were pooled.

Further purification of the pooled fractions was achieved by passing them through a Sephacryl S-300 column (1 × 100 cm), calibrated against a set of MW markers. The column was equilibrated and eluted with 0.05 M carbonate buffer, pH 9.2. The MW of the conjugates was determined and the fractions corresponding to the conjugates were pooled and stored at 4°C for at least 2 months. The protein concentration in the pooled fractions was determined following the method of Bradford (10) and using solutions of known concentration of the respective carrier proteins as standards.

In order to prepare T4-protein conjugates with different T4-to-protein molar ratio, different amounts of T4 were conjugated with the same amount of rabbit gamma-globulins by using the same conjugation procedure.

Calculation of the T4-to-Protein Molar Ratio of the Conjugates

The T4-to-protein molar ratio (r) was calculated by measuring the protein concentration of the conjugate using the method of Bradford (10) as described above, and the radioactivity associated with the conjugate fraction. The following equation was used for the calculation:

$$r = [(R/R_0) \times H]/P$$

where R = cpm associated with the conjugate fraction, R_0 = total cpm loaded on the column, H = moles of T4 added in the reaction mixture, and P = moles of protein.

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Immobilization of the T4-Protein Conjugates on the Solid-Phase

Microtitration wells were coated with T4-protein conjugates by adding 0.1 mL of T4-protein conjugate solution in 0.05 M carbonate buffer, pH 9.2, containing 0.5 g/L NaN_3. The concentration of each conjugate used for coating was appropriately selected in order to obtain similar zero standard absorbance readings (approximately 1.5 absorbance units) with all the different conjugates. After overnight incubation at RT, the wells were washed twice with 0.3 mL of 0.01 M *tris*-HCl buffer, pH 8.25, 0.5 g/L Tween 20 (washing buffer) per well. Then, the wells were blocked with 0.3 mL of 0.01 M phosphate buffer saline, pH 7.3, containing 50 g/L gelatin and 0.5 g/L NaN_3 for 2 h at RT and washed three times, as previously.

Influence of HSA on the Antibody Binding to Immobilized T4-Protein Conjugates

10 μL of zero standard or zero standard containing added amounts of HSA and 0.1 mL of 40 ng/mL MAb anti-T4 solution in 0.1 M phosphate buffer, pH 7.4, containing 10 g/L gelatin, and 0.5 g/L NaN_3 (assay buffer) were added per T4-protein conjugate coated well. The wells were incubated for 2 h at RT, under shaking, and then they were washed twice with 0.3 mL of washing buffer per well. After that, 0.1 mL of 2nd Ab-HRP solution (1 : 2000-fold dilution in 0.15 M *tris*-HCl buffer, pH 8.25, containing 1.0 g/L rabbit gamma globulins, 1.0 g/L BSA and 0.2 g/L Thimerosal) was added per well, and the wells were incubated for 2 h at RT under shaking. Following three washes of the wells, 0.1 mL of ABTS enzyme-substrate solution was added to each well. After incubation for 30 min at RT in the dark, the absorbance of the wells at 405 nm was measured using an ELISA reader (Multiscan RC, Labsystems OY, Finland).

Influence of HSA on the Antibody Binding to T4-Protein Radiolabeled Conjugates

T4-protein conjugates were radioiodinated with ^{125}I as described elsewhere.(9) The influence of HSA on the antibody binding to the radiolabeled conjugates was carried out by the following protocol: 30 μL of zero standard or zero standard containing added amounts of HSA were incubated with 0.1 mL of radioiodinated T4-conjugate and 0.1 mL of MAb anti-T4 solution, 60 ng/mL in assay buffer, for 30 min at RT. Then, 1.0 mL of goat anti-mouse antiserum, diluted 33-fold with 0.15 M *tris*-HCl buffer, pH 8.25,

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containing 100 mg/L mouse gamma globulins, was added and the mixture was incubated at RT for 15 min. After centrifugation at $1850 \times g$ for 15 min, the supernatant was decanted and the radioactivity of the precipitate was measured.

RESULTS AND DISCUSSION

The objective of this work was to investigate the effect of HSA concentration on the binding of anti-T4 antibody to immobilized T4-protein conjugates, with respect to the MW and the T4-to-protein molar ratio of the conjugates. T4-protein conjugates of different MW were prepared and used either as solid-phase reagents, or as liquid-phase radiolabeled analogs, in order to gain insight information concerning the properties of the immobilized conjugates, as compared to the properties of the liquid-phase labeled conjugates.

Rabbit gamma globulins, thyroglobulin, ferritin, and transferrin were used as carrier proteins for the preparation of the T4-protein conjugates. The MWs of the conjugates prepared are presented in Table 1. As it is shown, the MW of T4-thyroglobulin and T4-ferritin were equal to the MW of the carrier proteins. On the other hand, the T4-rabbit gamma-globulin conjugate seemed to be a polymer, since its MW was about seven times greater than the MW of rabbit gamma-globulin. Concerning the T4-transferrin preparation, we found that two conjugates of different MW were formed. The first one (T4-Transferrin-1) corresponded to a transferrin heptamer with a MW of 5.6×10^5 Da, whereas the second one (T4-Transferrin-2) was a transferrin dimmer (MW $= 1.6 \times 10^5$ Da).

The influence of HSA concentration on the binding of anti-T4 antibody to immobilized T4-protein conjugates was investigated using thyroid

Table 1. Molecular Weight of T4-Protein Conjugates Prepared Using Different Carrier Proteins

T4-Protein Conjugate Carrier Protein	MW of the Carrier Protein ($\times 10^5$ Da)	MW of the Conjugate ($\times 10^5$ Da)
Rabbit gamma-globulin	1.5	10
Thyroglobulin	6.7	6.7
Transferrin-1 (heptamer)	0.80	5.6
Ferritin	4.0	4.0
Transferrin-2 (dimer)	0.80	1.6

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hormone-free human serum (zero standard) spiked with increasing amounts of thyroid hormone stripped HSA. The signals obtained with these preparations were expressed as percentages of the zero standard signal. As it is shown in Figure 1, by increasing the serum albumin concentration from 40 g/L (zero standard) to 120 g/L, the observed signal decrease was less than 10%. This was true even for the conjugates with MW lower than 6.5×10^5 Da. This finding differs from what we reported in our previous work, concerning the use of T4-conjugates as liquid-phase tracers.(9) In this case, we had mentioned that, when the MW of the T4-protein conjugates was less than 6.5×10^5 Da, the binding potential of serum albumin for the conjugates was high and affected considerably the binding of anti-T4 antibody to the conjugates. In order to elucidate the reason of such a discrepancy, we compared the influence of albumin on antibody binding to conjugates, which were used either as immobilized or as liquid-phase reagents, with respect to the MW of the conjugates.

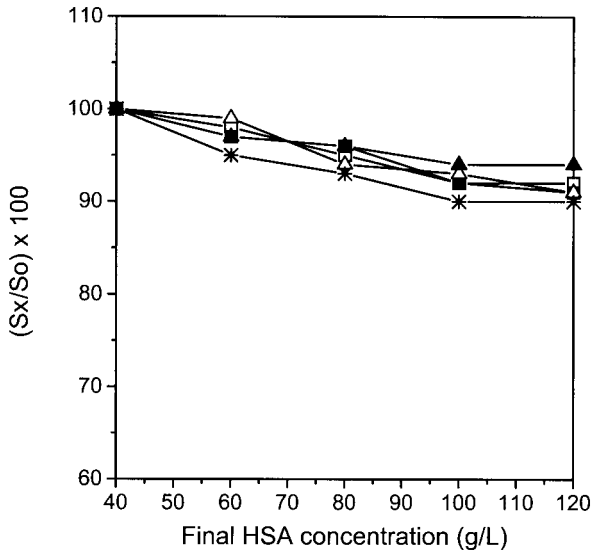

Figure 1. Influence of HSA concentration on the antibody binding to immobilized T4-protein conjugates. The signals (Sx) obtained after addition of HSA in zero standard were expressed as percentages of the zero standard signal (So). The concentration of each conjugate used for coating was 2.5 μg/mL for T4-rabbit gammaglobulin (▲), 5.0 μg/mL for T4-Ferritin (✳) and 10 μg/mL for T4-Transferrin-1 (□), T4-Transferrin-2 (■) and T4-Thyroglobulin (Δ). The concentration of the anti-T4 antibody used in all cases was 40 ng/mL.

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As it is shown in Figure 2, using the immobilized T4-protein conjugates, the signal decrease observed at a final HSA concentration of 80 g/L was 4–9%, irrespective of the MW of the conjugate ($y = 93.6 + 0.14x$, $r = 0.22$, $P = 0.73$). Using the same conjugates as liquid-phase tracers, we found that the signal decrease ranged from 33% (T4-Transferin-2, $1{,}6 \times 10^5$ Da) to 2% (T4-rabbit gamma globulin conjugate, $1{,}0 \times 10^6$ Da), and it was inversely proportional to the MW of the conjugates ($y = 61.8 + 3.58x$, $r = 0.995$, $P < 0.001$). These results clearly indicate that the influence of albumin was independent of the MW of the immobilized conjugates, whereas a strong dependence was observed when the conjugates were used as liquid-phase labeled analogs.

In addition to the MW of the conjugates, the T4-to-protein molar ratio of the conjugate was also examined as another parameter that could possibly affect the influence of albumin on the antibody binding to the immobilized conjugates. For this purpose, we prepared T4-rabbit gamma-globulin conjugates with different T4-to-protein molar ratio ranging from 0.24 to 3.8 moles of T4 per mole of protein. As it is shown in Figure 3, the signals obtained using final HSA concentrations of 80 and 120 g/L in the

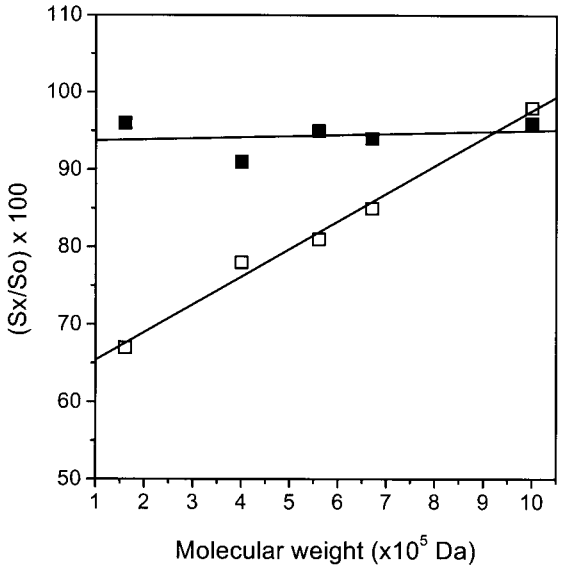

Figure 2. Correlation of the signal (Sx) obtained at a final HSA concentration of 80 g/L, expressed as percentage of the zero standard signal (So), against the MW of the immobilized T4-protein conjugates (■) and the liquid-phase labeled T4-protein conjugates (□).

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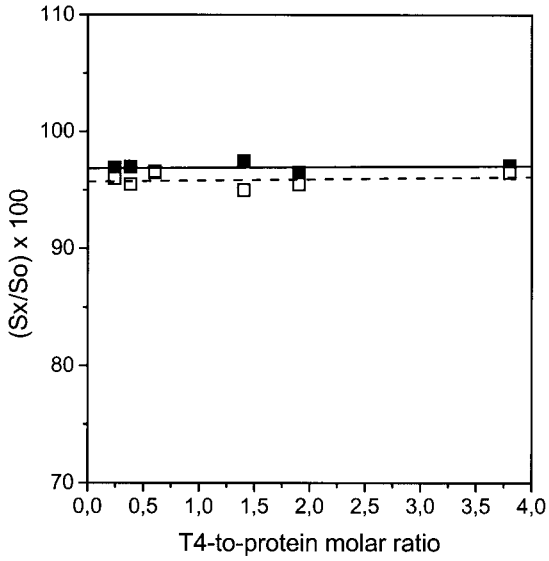

Figure 3. Correlation of the signal (Sx) obtained at 80 g/L (■, solid line) and 120 g/L (□, dashed line) final HSA concentration, expressed as percentage of the zero standard signal (So), against the T4-to-protein molar ratio of the conjugates used.

zero standard, were not correlated with the T4-to-protein molar ratio of the immobilized conjugates (HSA 80 g/L: $y = 96.8 + 0.055\,x$, $r = 0.19$, $P = 0.71$; HSA 120 g/L: $y = 95.7 + 0.11\,x$, $r = 0.23$, $P = 0.66$). In addition, in every case examined, the signals obtained were lower by less than 6% compared to the corresponding zero standard signals.

Current FT4 immunoassays seem to be improved, as compared to the former ones.(1,6,7) This improvement has been merely attributed to the use of T4-analogs as solid-phase reagents.(1,8) However, it is difficult to draw solid conclusions concerning the contribution of either the immobilization or the physicochemical characteristics of the T4-analogs to the improvement of FT4 immunoassays, based on information provided by kit manufacturers. This is due to the great diversity of assay formats, types of labeling, immunoassay reagents, and buffers used, that restricts direct comparisons in terms of the analogs used. In this work, we prepared T4-protein conjugates of different MWs and T4-to-protein molar ratios. We used these conjugates either as solid-phase reagents or as liquid-phase labeled analogs and we compared them under similar immunoassay conditions (same anti-T4 antibody and assay buffer). Our results indicated that the immobilized

T4-protein conjugates were superior to the corresponding liquid-phase analogs, since the antibody binding to the conjugates remained practically unaffected by the presence of albumin at concentrations as high as 120 g/L. Moreover, the immobilization of conjugates eliminated the strong dependency of the albumin effect on the MW of the conjugates that was encountered in case of liquid-phase labeled analogs. These findings support the "macroanalog" hypothesis, according to which the immobilized analogs may have substantially different properties from the same analogs in liquid-phase and may bind less to serum albumin.(1,8)

In conclusion, the results presented here indicate that the use of immobilized T4-analog approach in FT4 immunoassays is advantageous over the liquid-phase approach, especially concerning the issue of serum albumin concentration and/or affinity alterations. It is expected that the use of this approach for the development of FT4 immunoassays should help to further improve the reliability of FT4 determinations.

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Received May 17, 2001
Accepted June 24, 2001
Manuscript 3038

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