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ASSAY PERFORMANCE AND CLINICAL VALIDATION OF A RADIOIMMUNOASSAY FOR SERUM THYROXINE-BINDING GLOBULIN

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Summary: A radioimmunoassay for the quantitation of serum thyroxine-binding globulin (TBG), RIA TBG (PEG) – INEP, was formed and characterised. Test sensitivity was found to be favourable (2.0 mg/L). The highly satisfactory precision of the assay was reflected in the low coefficients of variation (less than 5%) found for both intra- and interassay precision at three concentrations. Non-specific binding of the labelled antigen was less than 2% of the total binding. Linearity of the assay within the range of the standard curve (0–80.0 mg/L) was excellent (r = 0.99) and the analytical recovery of TBG added to serum ranged from 91 to 115%. Reproducibility was checked by determining the concentration of TBG in samples of sera over a period of 32 days from the day of antigen iodination and $F<F^{0.05}_{tab}$, $F^{7,32}_{0.05} = 2.31$. The assay was standardised for accuracy using Lyphochek Immunoassay Controls (Bio-Rad). A significant correlation was obtained between RIA TBG (PEG) and the Kodak Amerlite TBG Assay (r = 0.84; n = 25). The presented results show that RIA TBG (PEG) is a sensitive, precise and reliable method for determination of TBG in human sera. Assay reference ranges were established for sera from various subjects: euthyroid adults, hypothyroid and hyperthyroid patients and in healthy pregnant women.

Key words: thyroxine-binding globulin (TBG), radioimmunoassay, reference intervals

Introduction

Thyroxine-binding globulin (TBG) is the most important plasma protein for transport of the circulating thyroid hormones: thyroxine (T_4) and triiodothyronine (T_3). The serum concentration of TBG can vary significantly in response to genetic factors (1), diseases (2, 3), pregnancy (4), drugs and hormones (5), which affect total and, to some degree, free thyroid hormone concentrations. Therefore, direct determination of serum TBG concentration is clinically important for correct interpretation of the total T_4 values during evaluation of the thyroid status of patients.

Several analitycal techniques have been developed for measuring TBG concentration. Some of them, such as radioimmunoassay or enzyme immunoassay, are now commercially available and widely used in laboratory practice.

We have recently purified and characterized a TBG preparation from pregnancy sera (6, 7). This preparation was radioactively labelled and used as a tracer in our radioimmunoassay system, RIA TBG (PEG) (8). In this paper we describe the performance of this assay. We have also determined our reference ranges for TBG concentrations in healthy and diseased subjects.

Materials and Methods

Highly purified TBG was prepared from pooled sera from pregnant women by a two step chromatografic procedure (6) and characterized (6, 7).

The preparation of specific rabbit anti-TBG antiserum was previously described (6).

Standard TBG serum. Sera from pregnant women (last trimester), diluted with 0.05 mol/L phosphate buffered saline (PBS) containing 0.25% bovine serum albumin (BSA), were used for preparation of

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the TBG standards. The range of the standard curve was from 0 to 80.0 mg TBG/L.

Chemiluminescent immunoassay. Kodak Amerlite TBG Assay (Amersham Kodak Clinical Diagnostics, UK) was performed according to the manufacturer's instructions and used for comparing TBG measurement results with the values obtained in our assay.

Subjects. Serum samples were obtained from 102 euthyroid subjects, 33 patients with hypothyrosis, 66 patients with hyperthyrosis and 346 healthy pregnant women.

Sera from euthyroid subjects were collected from healthy blood donors (Blood Transfusion Centre of Zemun Clinic) without any thyroid disorder. Samples from hypothyroid and hyperthyroid patients were obtained from the Department of Nuclear Medicine, Medical Centre Zaječar. The thyroid status of these patients was established on the basis of clinical evaluation and the measurement of TSH by an ultrasensitive TSH fluoroimmunoassay (Delfia Pharmacia, Sweden). Sera from healthy pregnant women with no history or symptoms of thyroid disorders as well as data about gestation stage were obtained from the Gynaecological Department of Zemun Health Centre. All samples were stored at –20 °C until analysis.

Assay procedure. RIA TBG (PEG) is a competitive immunoassay in which TBG standards and samples (diluted 1:11) and ^{125}ITBG compete for the limited amounts of binding sites on the specific polyclonal antibodies. After incubation overnight, at room temperature, PEG solution containing second antibody was added and the mixture incubated for 30 minutes at room temperature. The mixture was then centrifuged for 30 minutes at 3000 × g, the supernatant was decanted and the radioactivity of the precipitate was measured. The ^{125}I counts are inversely proportional to TBG concentrations in the standards and unknown samples.

Statistics. All statistical operations were performed using the STATGRAPHICS programme, version 4.2 (Student's T-test, analysis of variances, linear regression). Medians were analysed by Mann-Whitney U test. A value of Z higher than 1.96 ($p = 0.05$) was considered statistically significant. In accordance with the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) the central 95% range between the 2.5th and the 97.5th percentiles was taken to be the reference interval (9).

Results and Discussion

Assay characteristics

The detection limit of the assay was calculated from the mean radioactivity minus three standard deviations of the zero standard for 10 replicate analyses measured in an intraassay mode and found to be 2.0 mg/L. This detection limit obtained for our assay is comparable with previously published data for TBG immunoassays (about 3 mg/L) (10–12) and it is low enough to satisfy clinical needs for quantitation of TBG in the serum of TBG deficient subjects.

Table I Precision of RIA TBG (PEG)

Samples	n	Mean, mg/L	SD, mg/L	CV %
intraassay				
1	20	15.02	0.194	1.3
2	20	21.39	0.474	2.2
3	20	42.20	2.067	4.9
interassay				
1	20	14.70	0.609	4.1
2	20	22.98	0.991	4.3
3	20	37.61	1.260	3.4

Table II Dilution linearity of RIA TBG (PEG)

Sample, mg/L	Dilutions	Expected, mg/L	Measured, mg/L
80.0	1:11	80.0	74.0
	1:22	40.0	40.4
	1:44	20.0	21.0
	1:88	10.0	10.8
	1:176	5.0	7.7

Table III Analytical recovery of TBG added to serum sample

Sample, mg/L	Added, mg/L	Expected, mg/L	Measured, mg/L	Recovery, %
21.6	–	–	21.6	–
	10.0	31.6	33.1	115
	20.0	41.6	43.8	111
	40.0	61.6	58.0	91

The precision of the assay was evaluated by analysis of three different TBG concentration ranges. As summarised in *Table I*, intraassay CVs were 1.3–4.9% (10 duplicates). Interassay CVs determined in twenty successive runs ranged from 3.4 to 4.3%. An assay is acceptable if the coefficient of variation of the intraassay is less than 10% and of the interassay less than 15% (13). Thus, the assay precision is acceptable and compares well with that for other TBG radioimmunoassays: intraassay CV 5.6% (14), <5% (15); interassay 6.4% (14), <10% (15).

Non-specific binding of the labelled antigen, determined both in the sample and in the sample diluting buffer, was very low – less than 2% of the total binding. In order to evaluate dilution linearity within the range of the standard curve (0–80.0 mg/L), a serum sample with a high concentration of TBG (80.0 mg/L) was diluted up to 176 fold with diluent buffer. The observed concentrations decreased linearly with increasing dilution and the correlation bet-

Table IV Reference intervals of TBG determined by RIA TBG (PEG) in various subjects are given as the central 95% range (2.5th, the median, 97.5th percentiles)

Subjects		TBG, mg/L		
		2.5th	Me	97.5th
Euthyroid (n = 102)		18.5	25.6	32.2
Hypothyroid (n = 33)		22.5	29.0*	51.7
Hhyperthyroid (n = 66)		17.3	24.9	37.9
Pregnant women (n = 346)	1st trimester (n = 102)	23.3	36.7*	62.2
	2nd trimester (n = 107)	23.1	47.1*	66.6
	3rd trimester (n = 137)	22.8	53.5*	77.1

*Statistically significant difference (Z>1.96; p = 0.05) when comparing hypothyroid to euthyroid, pregnant women of 1st trimester to control group (57 non-pregnant women), 2nd trimester to 1st trimester and 3rd trimester to 2nd trimester.

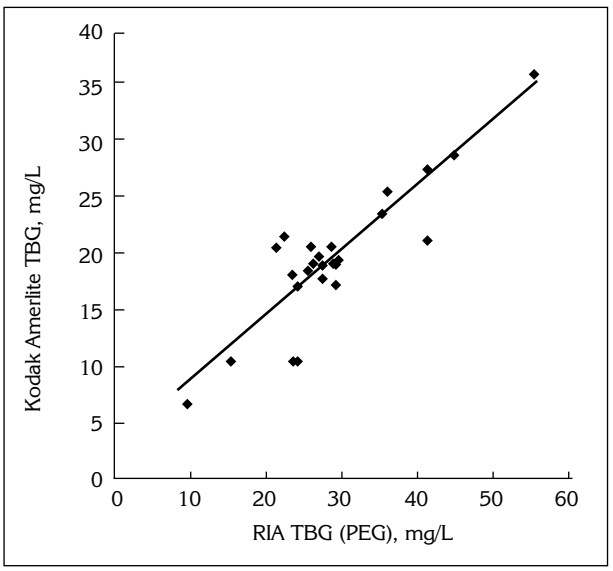

Figure 1 Regression analysis of the comparison between the RIA TBG (PEG) and Kodak Amerlite TBG Assay (y = 4.3021 + 0.5284x, r = 0.84, n = 25)

ween the expected and observed values was excellent (y = 3.0167 + 0.8956x, r = 0.99), (*Table II*).

The analytical recovery was assessed by adding increasing amounts of the standard TBG serum to a human serum and the TBG concentrations were assayed. The results are presented in *Table III*. The recoveries of TBG ranged from 91 to 115% and an excellent correlation between the expected and observed values was obtained (y = 8.4423 + 0.8128x, r = 0.99).

We also studied the effect of lipemia on the performance of the assay. Serial dilutions of lipemic sera with added TBG standard were assayed. No significant correlation between expected and measured values was obtained (r = 0.77, $t<t_{tab}$). Thus, TBG measurement in lipemic sera should be avoided. Lipemia probably impairs precipitation assays (such as RIA) while it did not cause any problems in a kinetic assay (10).

The assay was checked for accuracy using Lypochek Immunoassay Controls (Bio-Rad) at three levels. Our results were within the ranges obtained with other RIA kits (Biocode RIA and Brahms Diagnostica DYNOtest).

Reproducibility of the test was checked by determining the concentration of TBG in two samples of sera in eight runs over a period of 32 days from the day of antigen iodination. Analysis of variances showed that there were no significant differences between compared runs ($F<F^{0.05}_{tab}$, $F^{7,32}_{0.05} = 2.31$).

The TBG concentrations in 25 clinical serum samples were assayed in duplicate by our assay and the commercial Kodak Amerlite TBG Assay. As shown in *Figure 1*, the TBG values obtained by RIA TBG (PEG) compared well with those for the other assay and the correlation was statistically significant (y = 4.3021 + 0.5284x, r = 0.84).

Reference intervals for TBG

In order to define the reference intervals, the serum TBG concentration was determined by RIA TBG (PEG) in various groups of subjects (*Table IV*). TBG values for 102 euthyroid individuals (45 males and 57 females) ranged from 18.5 to 32.2 mg/L (median 25.6 mg/L). No difference was found in TBG concentrations between male and female subjects. The range of normal values was similar and comparable to those determined by other tests: 14.4–28.0 mg/L (11), 15.0–25.0 mg/L (12), 12.7–26.6 mg/L (16), although lower values have been reported by some investigators (17, 18).

Serum TBG concentration is under the complex influence of thyroid hormones and changes in its level have been observed in hypothyrosis and hyperthyrosis (19). We found that TBG values in 66 hyperthyroid patients were slightly but not significantly lower than those in euthyroid subjects (17.3–37.9 mg/L, median 24.9 mg/L), while those in 33 hypothyroid individuals were significantly elevated (22.5–51.7 mg/L, median 29 mg/L). Previously published

results for TBG in hyperthyrosis are generally not in agreement. Some authors found similar fluctuations (20) while significantly lower TBG values were reported by others (14, 21).

The increase of serum TBG associated with pregnancy requires the determination of trimester specific reference ranges. TBG concentration was measured in 346 healthy pregnant women and the central 95% reference range is shown in *Table IV*. TBG values in the first, second and third trimester ranged from: 23.3–62.2 mg/L; 23.1–66.6 mg/L and 22.8–77.1 mg/L, respectively. Statistical analysis revealed a significant rise of TBG concentration throughout the whole pregnancy ($Z>1.96$; $p = 0.05$).

In conclusion, we have shown that the presented RIA TBG (PEG) – INEP assay is a sensitive, precise and reliable method for TBG measurement in human serum. The reference intervals for TBG in euthyroid subjects, hypothyroid and hyperthyroid patients, as well as during pregnancy were also established.

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KARAKTERISTIKE I KLINIČKA VALIDACIJA RADIOIMUNOLOŠKOG TESTA ZA MERENJE TIROKSIN-VEZUJUĆEG GLOBULINA U SERUMU

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Kratak sadržaj: U laboratoriji INEP-a formiran je radioimunološki test za određivanje koncentracije tiroksin-vezujućeg globulina (TBG) u serumu i ispitane su njegove karakteristike. Osetljivost testa je 2,0 mg TBG/L. Preciznost određivanja koncentracije TBG-a u tri uzorka seruma u seriji i između serija je veoma velika (KV < 5%). Nespecifično vezivanje obeleženog antigena ne prelazi 2% ukupnog vezivanja. Linearnost testa u opsegu standardne krive (0–80 mg/L) je odlična (r = 0,99) dok je kod metode dodatog standarda dobijen »recovery« od 91 do 115%. Pokazana je stabilnost testa u roku od 32 dana od dana jodovanja TBG-a ($F<F^{0,05}_{tab}$, $F^{7,\ 32}_{0,05} = 2,31$). Tačnost testa je ispitana korišćenjem međunarodnih kontrola za standardizaciju Lyphochek Immunoassay Controls (Bio-Rad). Dobijen je značajan stepen korelacije između RIA TBG (PEG) i Kodak Amerlite TBG Assay (r = 0,84; n = 25). Dobijeni rezultati ukazuju da je RIA TBG (PEG) precizna, osetljiva i pouzdana metoda. Takođe su određene referentne vrednosti TBG-a kod eutireoidnih, hipotireoidnih i hipertireoidnih osoba kao i kod trudnica.

Ključne reči: tiroksin-vezujući globulin (TBG), radioimunološki test, referentne vrednosti

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