COMPARISON OF SERUM THYROGLOBULIN MEASUREMENTS BY RADIOIMMUNOASSAY AND IMMUNORADIOMETRIC ASSAY

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ABSTRACT

Serum thyroglobulin (Tg) concentrations are widely used as a tumour marker for monitoring the patients with differentiated thyroid carcinoma. Precise and reproducible Tg measurements are critical, especially when patients are judged to have a high risk for recurrence. The clinical utility of two different Tg methods (RIA and IRMA) was compared and evaluated focusing on measurement methodology. Therefore, complete quality control profiles were performed and assessed. The results revealed that the sensitivity (low detection limit) of the assays for Tg-RIA (DPC) and Tg-IRMA (CIS) was 2.28 ng/ml and 0.62 ng/ml, respectively. The assay precision of both intra-and inter-assays had coefficient of variation (C.V.) of 5.49-15.63% for Tg-RIA and 5.56-8.27% for Tg-IRMA. The accuracy of the two assays was 96.9-121.3% for Tg-RIA and 97.6-104.3% for Tg-IRMA. No cross-reaction and no drift effect were obtained in both assays. The results showed complete parallelism between Tg standards and serial dilutions of Tg-containing serum. The hook effects were indicated with both assays at very high concentration of Tg. The quality of Tg-IRMA (CIS) was proved to be superior to Tg-RIA (DPC).

Quantitation of thyroglobulin (Tg) in the circulation is considered to be a good diagnostic test for the detection of the presence of metastases or recurrence of differentiated thyroid carcinoma (papillary and follicular).[1,2,3,4,5,6] It is well established that serial Tg measurements are usefully employed in the management of the patients following removal of the thyroid gland by surgery and radioablation, if successful, should result in circulating Tg stabilizing at very low or undetectable levels; higher levels, on the other hand, are suggestive of remnent thyroid tissue or metastasis. Numerous methods have been developed to measure Tg in serum, including radioimmunoassay (RIA), immunoradiometric assay (IRMA) and enzyme immuno-assay.[7,8,9,10,11,12,13]

The detection limit is an important characteristic because the main interest of the assay is the follow-up of differentiated thyroid cancer. It is critical to be able not only to detect small amounts of Tg, but also to observe a change in Tg concentration. Thus the objective of this study is to compare the two commercial assays for thyroglobulin : a competitive-binding RIA by Diagnostic Products Corporation (DPC) and the 'CIS' IRMA-type assay utilizing quality control (Q.C.) profiles such as sensitivity, precision, accuracy, specificity, drift test, parallelism and hook effect.

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MATERIALS AND METHODS

BLOOD SAMPLES FOR DILUTION TEST AND HOOK EFFECT

Very high Tg concentrations from the sera of patients with differentiated thyroid carcinoma were selected, mixed and pooled. The serum mixture was then made to the dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 for the test of hook effect.

STOCK AND WORKING Tg STANDARDS

One milligram of purified Tg from human thyroid glands was kindly obtained from Dr. S. Damrongpisuttikul,[14] weighed and dissolved in a few drops of 10.0 M sodium hydroxide, and then diluted to 100 ml with 0.05 M phosphate buffer pH 7.4 containing 0.05%BSA to give the concentration of Tg about 10,000 ng/ml. For the hook effect test, the stock Tg solution was further diluted to the serial two-fold dilutions with the 0.05 M phosphate buffer pH 7.4 giving the working Tg standard of 0-5,000 ng/ml.

MEASUREMENT OF SERUM Tg

1. TG RIA (DIAGNOSTIC PRODUCTION CORPORATION OR DPC, U.S.A.)

The serum Tg was measured by a double antibody (Donkey anti-goat gamma-globulin) and diluted polyethylene glycol (PEG) in saline as precipitating agent. 200 µl of the zero calibrator for maximum binding tubes and for non-specific binding (NSB) tubes, Tg standards at different concentrations,Q.C.sera at low and medium concentrations, and unknown samples were pipetted into 12×75 mm polypropylene tubes. 100 µl Tg antiserum was added to all tubes except the 'NSB' tubes and mixed. After 2 hours incubation at room temperature, 100 µl of ^{125}I-Tg was added, mixed and incubated for 2 hours at room temperature. 1.0 ml of the cold precipitating solution was added, mixed and incubated for 30 minutes. The tubes were centrifuged at 3,000 g for 15 minutes. Supernatant was discarded and counted the precipitate in a gamma counter. Tg concentrations for the unknown samples were read out from the calibration curve.

2. TG IRMA (CIS-BIOINTERNATIONAL, FRANCE)

The assay was performed by dispensing 300 µl of the buffer into each ELSA-tube. 100 µl of Tg standards, controls and serum samples was added and mixed on a horizontal shaker for 3 hours at room temperature. The tubes were then washed twice with 3 ml of the washing solution. After addition of 300 µl of ^{125}I anti-Tg monoclonal antibody, the tubes were incubated for 18-24 hours at room temperature and then washed twice with 3 ml of the washing solution. The radioactivity bound to the tubes was measured in a gamma counter. The concentrations of the samples were read directly from the standard curve.

STATISTICAL ANALYSIS

The mean, standard deviation, percentage and coefficient of variation (CV) were determined by the program of SPSS 7.5 for window.[15]

RESULTS

The validation of Tg RIA and Tg IRMA was carried out and compared as follows :

1. RESPONSE STANDARD CURVE

The typical standard curves of both assays were constructed and shown in Figures 1 and 2.

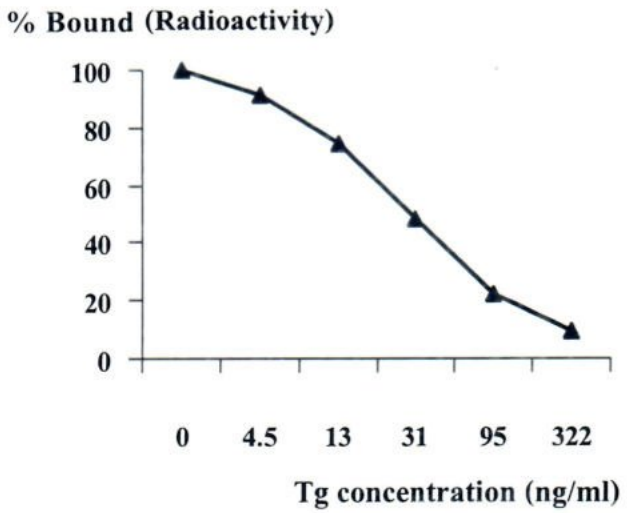

Fig. 1. Response standard curve for Tg RIA (DPC)

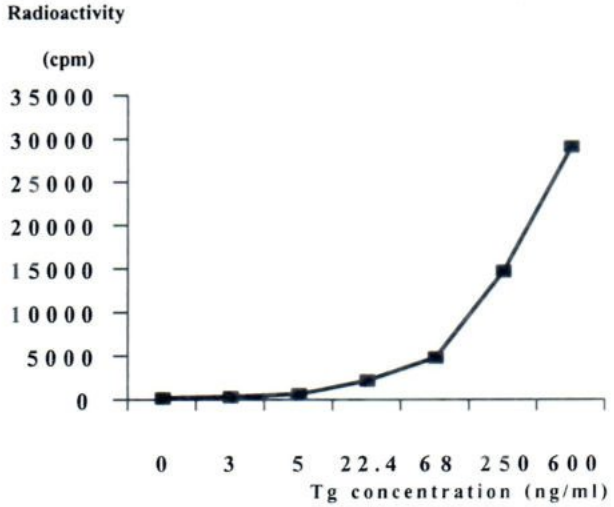

Fig. 2. Response standard curve for Tg IRMA (CIS)

2. SENSITIVITY TEST

Twenty zero calibrator tubes were processed in a single assay, along with a set of nonzero calibrators. Mean and standard deviation were calculated and two standard deviations were subtracted from mean counts at zero point. The detection limit (minimal detectable dose) of Tg RIA and Tg IRMA was 2.28 ng/ml and 0.62 ng/ml, respectively.

3. PRECISION TEST

The reliability of 2 assay kits was performed by examining their reproducibility on control sera to represent a range of Tg levels. The coefficient of variation (C.V.) was determined for each of two control sera from the results of 20 pairs of tubes in a single assay for intra-assay precision and in 20 different assays for inter-assay precision. Better inter-assay reproducibility was obtained from the IRMA Tg kits (CIS) as summarized in Tables 1 and 2.

4. ACCURACY TEST

Known amounts of Tg were added to human sera and then assayed. The accuracy of the test was expressed in percentage of recovery (observed value / expected value). Analytical recovery varied between 96.9%-121.3% for the RIA Tg kits and 97.6%-104.3% for the Tg IRMA as provided in Tables 3 – 4.

Table 1. Assay reproducibility of Tg RIA

Assay precision	Tg RIA (DPC)			
	No.	Mean	SD	%CV
Intra-assay				
Control serum 1	20	8.80	0.48	5.49
Control serum 2	20	62.57	3.62	5.78
Inter-assay				
Control serum 1	20	9.72	1.52	15.63
Control serum 2	20	63.22	8.16	12.90

Table 2. Assay reproducibility of Tg IRMA

Assay precision	Tg IRMA (CIS)			
	No.	Mean	SD	%CV
Intra-assay				
Control serum 1	20	7.81	0.43	5.56
Control serum 2	20	48.49	2.82	5.78
Inter-assay				
Control serum 1	20	8.48	0.70	8.27
Control serum 2	20	50.43	3.73	7.39

Table 3. The percentage of recovery for the Tg RIA (DPC)

Number of samples	Observed value (ng/ml)	Expected value (ng/ml)	%Recovery
1	11.6	11.4	101.8
2	21.7	22.4	96.9
3	52.6	53.2	98.9
4	37.0	30.5	121.3
5	46.0	39.8	115.6
6	78.0	74.0	105.4
		Mean ± SD = 106.7 ±9.7	

Table 4. The percentage of recovery for the Tg IRMA (CIS)

Number of samples	Observed value (ng/ml)	Expected value (ng/ml)	%Recovery
1	2.1	2.0	103.5
2	5.7	5.5	104.2
3	14.6	14.0	104.3
4	44.9	46.0	97.6
5	136.4	138.0	98.8
6	497.5	490.0	101.5
		Mean ± SD = 101.7 ±2.9	

5. SPECIFICITY TEST

The quality of Tg antibodies for two commercial kits was assessed by cross-reactivity tests with monoiodothyronine (T_1), diiodothyronine (T_2), triiodothyronine (T_3), thyroxine (T_4) and thyrotropin (TSH). The results indicated that the Tg antibodies used in the both assays did not present any cross-reaction with these analogues as illustrated in Figures 3 and 4

RADIOACTIVITY OF

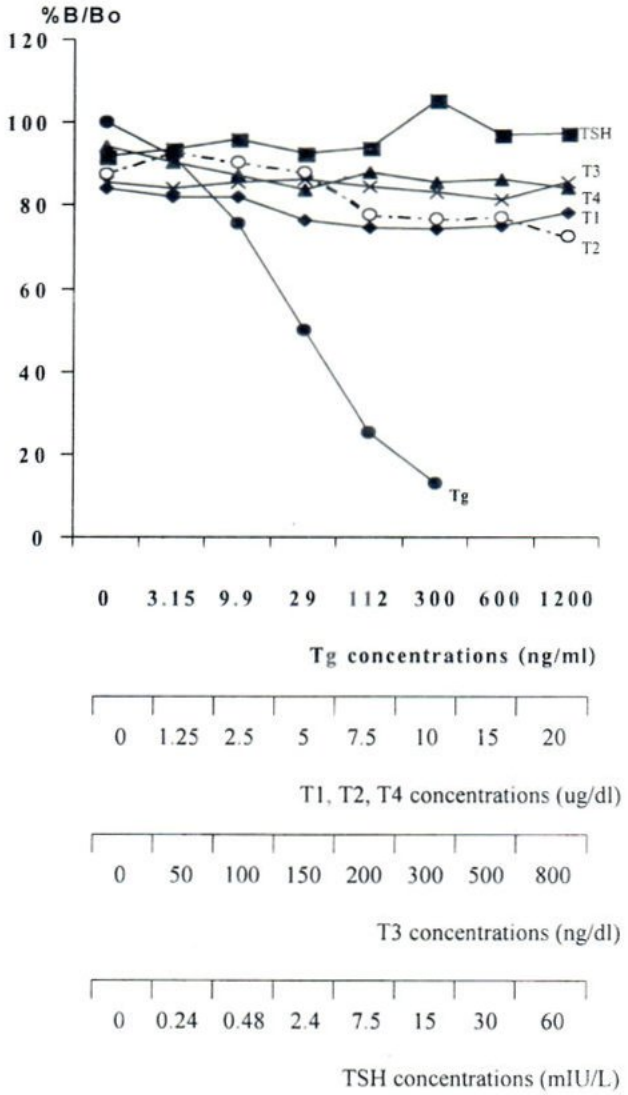

Fig. 3. Specificity of Tg antibody for Tg RIA (DPC)

6. PARALLELISM TEST

This test was carried out by adding the serum sample (A) to each concentration of the

standard Tg, giving a final dilution of 1:1. The two response curves were plotted and compared. Parallelism between sample and standard of the two methods was observed as seen in Figures 5 and 6.

7. DRIFT TEST

Pairs of three different Tg concentra-tions were spaced throughout a long assay of RIA and IRMA. There were any position effect due to delay in the addition of the reagents as listed in Tables 5 and 6.

Table 5. Drift test for Tg RIA (DPC)

Tg RIA	Concentrations of Tg in the samples (ng/ml)		
	A	B	C
• Tube No.			
21 - 26	13.10	65.81	89.60
65 - 70	13.12	68.95	90.31
95 - 100	13.69	72.86	93.15
• Mean	13.30	69.21	91.02
• SD	0.27	2.89	1.53
• % CV	2.06	4.17	1.69

Table 6. Drift test for Tg IRMA (CIS)

Tg IRMA	Concentrations of Tg in the samples (ng/ml)		
	A	B	C
• Tube No.			
21 - 26	6.58	56.07	837.55
51 - 56	6.67	57.58	847.07
91 - 96	6.86	59.75	852.23
• Mean	6.70	57.80	845.62
• SD	0.11	1.51	6.08
• % CV	1.74	2.61	7.19

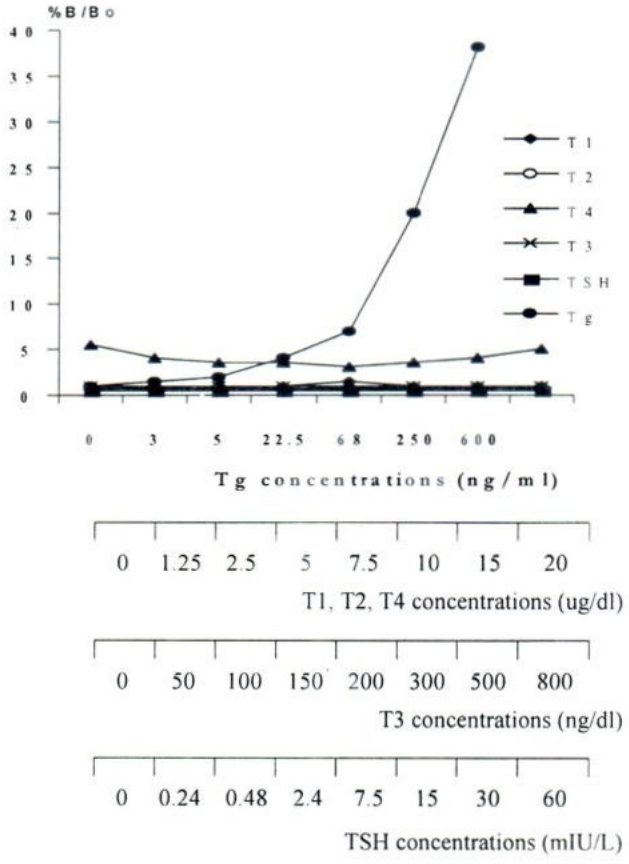

Fig. 4. Specificity of Tg antibody for Tg IRMA (CIS)

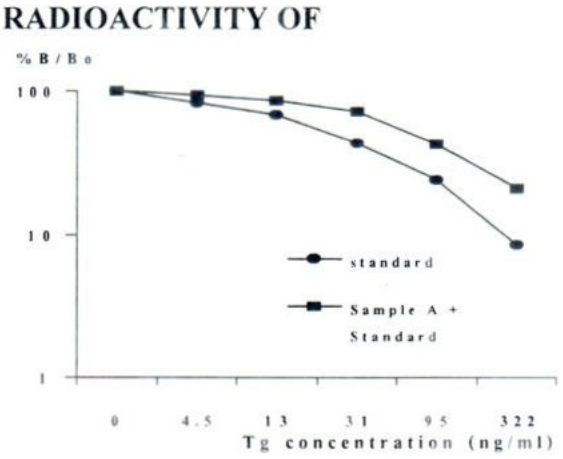

Figure 5. Parallelism test for Tg RIA (DPC

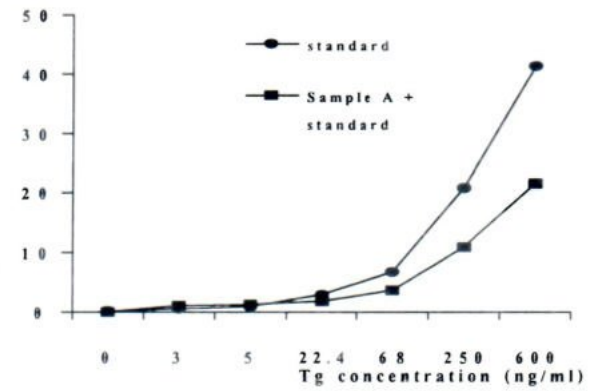

Fig. 6. Parallelism test for Tg IRMA (CIS)

8. HOOK EFFECT TEST

The tests were made in two selected patient sera with very high Tg concentrations utilizing the RIA and IRMA. In order to establish the final Tg values, serum Tg concentrations were determined in the undiluted and diluted sera at dilutions of 1:10, 1:100 and 1:1000. The hook effect results were given in Tables and Figures 7 and 8.

Table 7. Hook effect at different dilutions of the patient sera for Tg RIA

Serum dilutions	Tg concentrations (ng/ml)
Sample A	
Undiluted serum	390.68
1 : 10	2445.08
1 : 100	9151.00
1 : 1000	2017.30
Sample B	
Undiluted serum	395.11
1 : 10	2851.59
1 : 100	9601.10
1 : 1000	2679.30

Table 8. Hook effect at different dilutions of the patient sera for Tg IRMA

Serum dilutions	Tg concentrations (ng/ml)
Sample A	
Undiluted serum	480.11
1 : 10	6024.73
1 : 100	20739.10
1 : 1000	29438.00
Sample B	
Undiluted serum	423.76
1 : 10	4701.05
1 : 100	70368.40
1 : 1000	82193.00

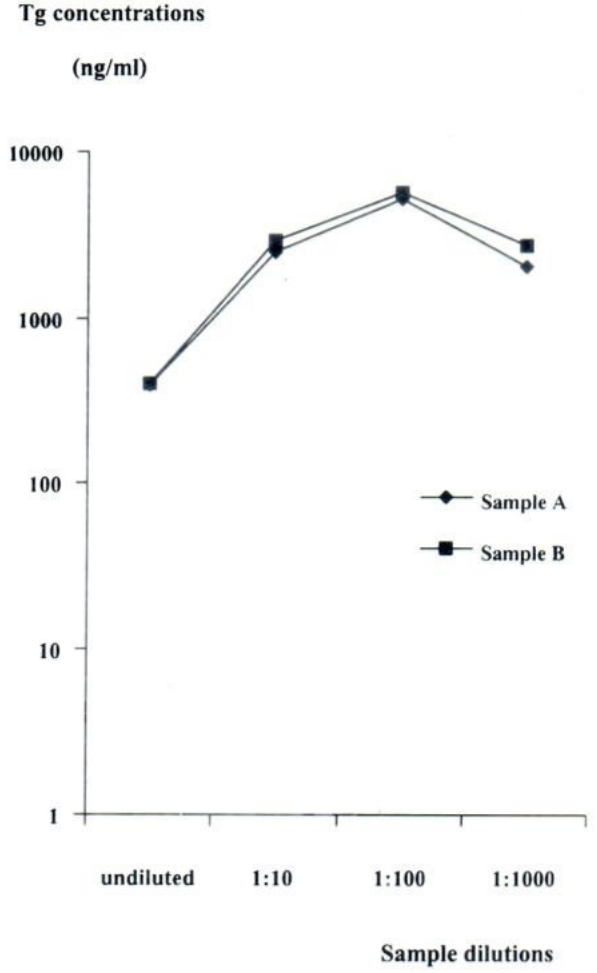

Fig. 7. Hook effect at different dilutions of Sample A and B for Tg RIA

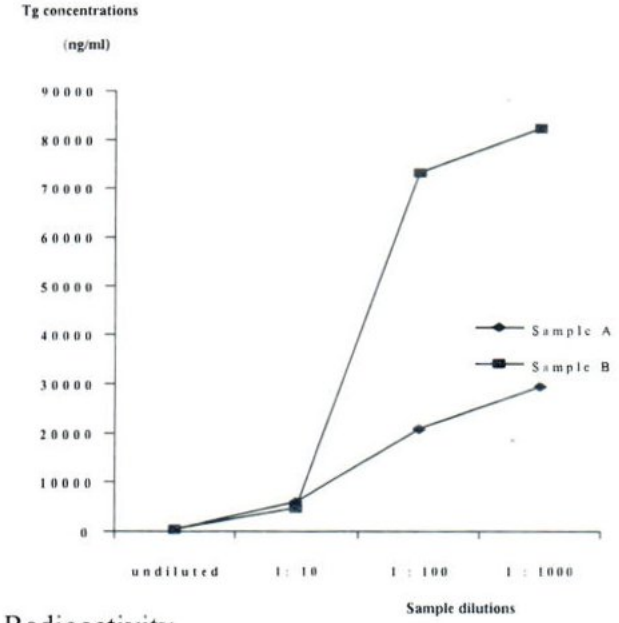

Fig. 8. Hook effect at different dilutions of Sample A and B for Tg IRMA

Purified preparations of human Tg were kindly offered by Dr. S. Damrongpisuttikul. Therefore, the standard curve of Tg at very high concentratios was performed by IRMA technique, and hook effect was obtained as illustrated in Figure 9.

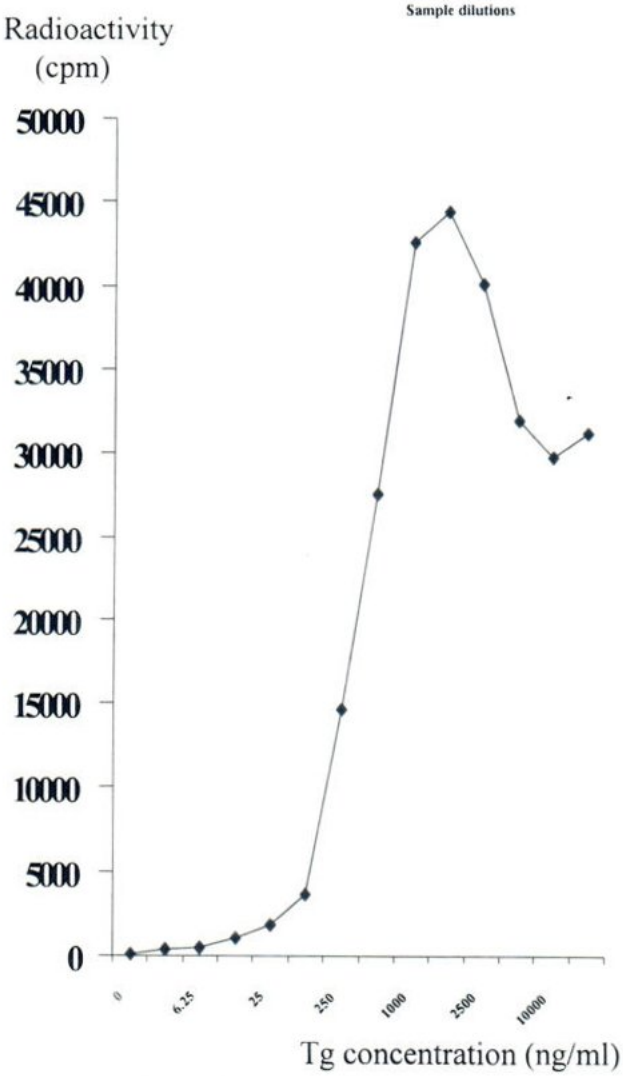

Fig. 9 Hook effect at high concentrations of standard Tg

DISCUSSION

The measurement of Tg in serum is technically challenging and now widely accepted as a sensitive and specific alterna-tive to radioiodine scans in the detection of residual, recurrent or metastatic disease in patients with differentiated thyroid cancer.[3,4,16] The first haemagglutination techniques were replaced by RIA.[7,8,9] Subsequently, monoclonal antibody technology has led to the development of IRMA[10,11] and ELISA.[12,13] Therefore, it is interesting to compare the quality of two commercial assays between Tg RIA (DPC) and Tg (CIS), and to choose the best one of the assay kit since incorrect results can lead to diasterous effects for the patients.

The typical response standard curves for a competitive-binding RIA by Diagnostic Production Co. (DPC) and a noncompetitive-binding IRMA by CIS bio international (CIS) were illustrated in Figures 1 and 2, respectively.

The minimal detectable concentration or sensitivity is an important characteristic because the main interest of the assay is the follow-up of patients with differentiated thyroid carcinoma, so it is critical to be able not only to detect small amounts of Tg but also to observe a change in Tg concentration.[17] The sensitivity was determined using the calculated error at zero concentration. Two standard deviations were subtracted from the mean counts at zero point and

the corresponding Tg concentration was read off from the standard curve. The sensitivity of Tg RIA (DPC) was found to be 2.3 ng/ml which were similar to those found earlier by previous authors.[5,18,19] The higher sensitivity was also reported by Ashcraft et al.[20] and Charles et al.[21] However, the lower sensitivity of Tg was obtained by the previous studies at 5.0 and 15 ng/ml.[22,23]

The Tg assay should be sensitive enough to detect concentrations as low as 1.0 to 2.0 ng/ml which can use in the follow-up of patients with differentiated thyroid cancer. The more sensitive assays are capable of distinguishing the lower limit of euthyroid range from the functional sensitivity limit. The sensitivity of Tg IRMA (CIS) was found to be 0.6 ng/ml giving the higher sensitivity than Tg RIA (DPC). Marquet and co-workers reported the highest sensitivity of 0.2 ng/ml for Tg IRMA[24] but the lowest sensitivity of 3.0 ng/ml was presented by the previous authors.[25,26]

The reproducibility or precision is the error associated with assay results. Therefore, the intra- and inter-assay precision was carried out using two control sera with different Tg concentrations. The coefficients of variations (CVs) of intra-assay precision were determined in the same series of Tg assays. The results of intra-assay CVs for Tg RIA (DPC) and Tg IRMA (CIS) were 5.49%-5.78% and 5.56%-5.78%, respectively. The inter-assay precision is a measure of variability associated with test results in different series of assays. The inter-assay CVs for Tg RIA (DPC) and Tg IRMA (CIS) were found to be 12.90%-15.63% and 7.39%-8.27%, respectively. The acceptable intra- and inter-assay CVs should be less than 10% for good precision of assay results which showed that Tg IRMA (CIS) had better assay precision than Tg RIA (DPC) especially inter-assay CVs as summarized in Tables 1 and 2.

Accuracy of the assay is the ability of an assay to detect all of the substances being assayed that is present in the sample. Analytical recovery test was made by adding known amounts of Tg to serum samples. The percentage of recovery of Tg RIA (DPC) and Tg IRMA (CIS) in six samples were 96.9%-121.3% with the mean (±SD) of 106.6 ± 9.7% and 97.6 – 104.3% with the mean of 101.7 ± 2.9%, respectively as given in Table 3 and 4. Better recovery results were also noted in Tg IRMA (CIS).

Specificity of the Tg antibodies was performed by cross-reactivity tests with different thyroid analogues such as T1, T2, T3, T4 and TSH. The results demonstrated that the antibodies used in the both assays did not present any cross-reaction with their analoques as illustrated in Figures 3 and 4.

In a valid assay, it is essential to test that the standard and unknown sample react identically with the same antibody binding sites. Thus, parallelism test between serum sample and standard was made because it can be done to assess interfering factors and for comparing the molecular integrity of the standard and sample. The results revealed that parallelism between the two curves of the both methods was observed as seen in Figures 5 and 6.

Drift test was also performed in order to determine whether there is any position effect due to delay in the addition of reagents. Three different concentrations of Q.C. sera were spaced throughout along the assays. There was no significant position effects in the both assays even in assays involving as many as 100 tubes (Tables 5 and 6).

The hook effect is referred as the phenomenon of a falling dose-response at very high analyte concentrations. The IRMA techniques were found to be more often subjected to hook effect than RIA methods.[17,27] The hook effect can lead to inappropriately low- or euthyroid-range Tg values in sera

with very high serum Tg concentrations, which require dilution. The hook effect appears to result when a massive excess of antigen (10 to 10,000 times the upper limit of the assay range) exhausts the binding capacity of the Tg capture antibody on the solid support. The hook effect test is necessary since concentration of tumour marker can be exceedingly high and the consequence of such an error has serious medical implication. [28] It is interesting to demonstrate whether these assays exhibited a high-dose hook effect in which a high concentration produced values lower than the value of the highest standard concentrations. Therefore, two selected serum samples with very high Tg concentrations were assayed in undiluted and diluted samples at 1:10, 1:100 and 1:1,000 dilutions to establish the final Tg value, which was based on the dilutions that produced parallelism within the assay working range. Hook effects were obtained in both different methods when Tg values were over 1,000 ng/ml as defined in Figures and Tables 7 and 8.

The purified preparation of human Tg was kindly provided by Dr. Sunetra Damrongpisuttikul[14] so a wider working range of the standard (0-10,000 ng/ml) could be performed according to Tg IRMA (CIS) method. A falling dose-response or hook effect occurred at very high Tg concentration of 1,250-2,500 ng/ml as presented in Figure 9.

Spencer and their co-workers[27] suggested the elimination of hook effect that the users of RIA methods should periodically validate the upper assay limit by diluting specimens having concentrations close to the upper limit with patients' specimens. Thus, the users of IRMA methods should run every serum specimen at two dilutions (undiluted and 1:10 dilution) to detect hook problems.

CONCLUSION

The quality of two different Tg methods was assessed and compared by using complete Q.C. profiles. This study revealed that different methods have different sensitivities which were 2.3 ng/ml for Tg RIA (DPC) and 0.6 ng/ml for Tg IRMA (CIS). The CVs of the assays both intra- and inter-assay precisions were 5.49-15.63% for Tg RIA (DPC) and 5.56-8.27% for Tg IRMA (CIS). The accuracy of the two assays was determined by recovery tests which were 96.9-121.3% for Tg RIA (DPC) and 97.6-104.3% for Tg IRMA (CIS). No cross-reaction between Tg antiserum and their analogues in both assays was observed. Parallelism between Tg standard and serial dilutions of Tg-containing serum sample was obtained, and no drift effects occurred in both assays. The hook effects of both assays were noted at very high Tg concentrations.

In conclusion, the quality of Tg IRMA (CIS) was proved to be superior to Tg RIA (DPC) for detecting residual, recurrent of metastatic patients with differentiated thyroid cancer.

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