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Unforeseen Effect of Thyroxine Binding Globulin when Using the Microencapsulated Antibody Method to Determine Free Thyroxine (FT_4): Misleading Results Due to Circulating Unsaturated Thyroxine Binding Globulin

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Summary: The effect of varying concentrations (0–52 mg/l) of purified thyroxine binding globulin (TBG) on the microencapsulated antibody method for free thyroxine was investigated. The results demonstrated that the free thyroxine values were strongly influenced by the concentration of thyroxine binding globulin in the samples. The standard curve could no longer be distinguished at a concentration of purified thyroxine binding globulin of 52 mg/l.

In the clinical application, we observed that the values obtained using the microencapsulated antibody method were significantly higher than the expected values in patients receiving triiodothyronine treatment after total thyroidectomy (theoretically nil) and in patients with untreated primary hypothyroidism with negligible thyroxine (less than 12.9 nmol/l). These false positive values are considered to be due to the methodological problem mentioned above, i.e. the microcapsule membrane is not efficient and therefore must be improved. Consequently, any data based on this method should be interpreted with caution.

Introduction

It is generally accepted that current free thyroxine (FT_4) radioimmunoassay kits routinely used in clinical laboratories are most useful for differentiating between healthy individuals and those with thyroid disorders (1, 2). Although differing considerably in both theory and practice, several methods for assaying FT_4 are available (3). However, a completely reliable method is still lacking. During the past decade, numerous papers have been published on the results obtained using these commercial methods (4–6). Unfortunately, as with all clinical laboratory methods, these methods suffer from interferences. The most common is the effect of abnormal albumin concentrations or non-esterified fatty acid and thyroxine binding globulin (5, 13). Thus far, only one paper has revealed in part that the results obtained with microencapsulated antibody methods were affected by circulating unsaturated thyroxine binding globulin (7).

In the present study, using the Liquisol FT_4 kit, the distortion of results by circulating unsaturated thyroxine binding globulin in the determination of FT_4 was investigated. This resulted in an evaluation of the clinical accuracy of the Liquisol FT_4 kit, in the diagnosis of severe primary hypothyroidism.

Materials and Methods

Experimental procedures

Preparation of thyroxine binding globulin

Highly purified thyroxine binding globulin was prepared from pooled human serum by affinity chromatography as previously described (8). The technique employed was essentially that described by *Pensky & Marchall* (9). [^{125}I]Thyroxine was added to the purified material and the mixture subjected to disc electrophoresis. The single band of stained protein coincided with the single peak of radioactivity. The thyroxine binding globulin did not contain any thyroxine (T_4) after purification.

Assay procedures

The assay for FT_4 using the Liquisol FT_4 kit (Damon Diagnostics, USA) was performed in full accordance with the manufacturer's instructions. Briefly, 25 µl of purified thyroxine binding globulin (0, 13, 52 mg/l) were added to test tubes containing 500 µl microcapsule suspension, which contained anti-T_4 combined with [^{125}I]thyroxine. Twenty-five µl of each standard (0, 1.94, 4.90, 14.2, 25.8, 40.0 and 64.5 pmol/l) were pipetted into correspondingly labelled tubes and each tube was vortexed for 4 seconds. The tubes were subsequently incubated in a water bath at 37 °C for 2 hours. After one hour, all the tubes were vortexed for a further 4 seconds and then returned to the incubator. After incubation, 1 ml of wash solution (polyethylenimine) was added to each tube, which was then vortexed for a further 4 seconds. The tubes were incubated at room temperature for 20 minutes, centrifuged at 1400 *g* for 10 minutes, then decanted. The microcapsules were counted in a gamma counter for 1 minute. In the range 5.16–58.1 pmol/l, the intra-assay coefficient of variation was 7.2–8.5%. In the range 12.9–38.7 pmol/l, the inter-assay coefficient of variation was 14.7–15.9%. FT_4 was also measured using the Amerlex kit (Amersham, U.K.); triiodothyronine, thyroxine, thyrotropin, thyroglobulin and thyroxine binding globulin were measured with the EIKEN kit (EIKEN ICL, Japan), and T_3 uptake (T_3U) was measured with the Dainabot kit (Dainabot, Japan). The accuracy of these kits and the analytical procedures for measurement of these hormones have previously been described in detail (7). All samples were measured simultaneously in order to avoid inter-assay variations. The results are expressed as the mean ± standard deviation (SD).

Subjects

Thyroid function studies were performed on 156 normal adult subjects, 106 pregnant women (30 subjects, 0–10 weeks; 22 subjects, 11–20 weeks; 24 subjects, 21–30 weeks; 30 subjects, 31–40 weeks), 26 hyperthyroid patients and 29 primary hypothyroid patients with detectable thyroxine (12.9–51.6 nmol/l). In addition, 16 patients with severe primary hypothyroidism with negligible thyroxine (less than 12.9 nmol/l) and 17 patients receiving triiodothyronine treatment after total thyroidectomy (thyroxine less than 12.9 nmol/l) were studied. Sera were aliquoted and stored at −20 °C until the assay.

Results

The effect of different concentrations (0–52 mg/l) of purified thyroxine binding globulin on the microencapsulated antibody method is shown in figure 1. Four test tubes were prepared, each containing 500 µl of microcapsule suspension. To the first was added 25 µl of serum containing no thyroxine binding globulin; to the second, 25 µl of serum containing 13 mg/l of purified thyroxine binding globulin; and to the third, 25 µl of serum containing 52 mg/l of purified thyroxine binding globulin. The results demonstrated that the FT_4 values, determined by Liquisol FT_4, were strongly influenced by the concentration of thyroxine binding globulin in the samples. When the thyroxine binding globulin concentration was increased, [^{125}I]thyroxine was extracted from the microcapsules. Due to the leakage of [^{125}I]thyroxine from the microcapsules, the standard curve can no longer be distinguished at a concentration of thyroxine binding globulin of 52 mg/l (fig. 1).

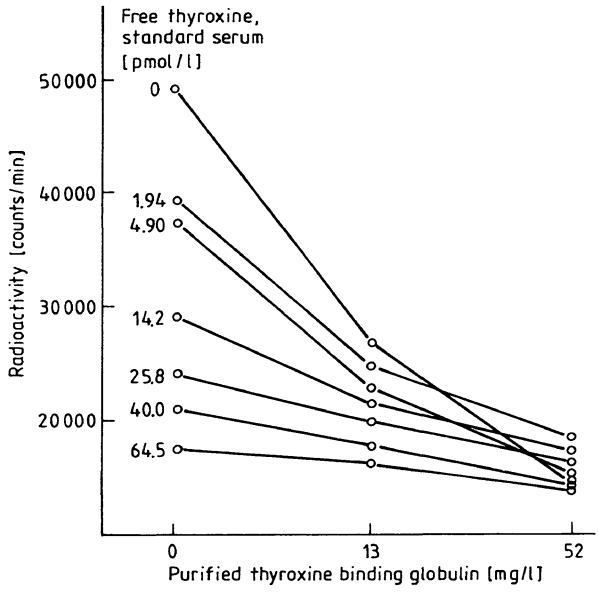

Fig. 1. Effect of thyroxine binding globulin on the microencapsulated FT_4 determination method.

A further examination of the Liquisol kit was undertaken as a clinical study. Table 1 shows the circulating FT_4 levels determined by the Amerlex kit and Liquisol kit in healthy normal subjects, pregnant women, patients with hyperthyroidism, and with primary hypothyroidism with detectable thyroxine. As shown in the table, the values obtained using the Liquisol kit were always higher ($P < 0.001$) than those from the

Tab. 1. Concentrations (mean ± SD) of serum FT_4 determined with the Amerlex kit and the Liquisol kit in normal subjects, patients with hyperthyroidism, hypothyroidism and pregnant women.

Subjects	n	Amerlex kit	Liquisol kit
Normal subjects	156	15.5 ± 3.2	21.9 ± 4.9 **
Hyperthyroidism	26	60.0 ± 18.2	72.5 ± 20.4 **
Hypothyroidism	29	2.97 ± 2.71	8.26 ± 3.35**
Pregnant women	106		
0–10 weeks	30	25.3 ± 5.9	30.4 ± 7.1 **
11–20 weeks	22	20.6 ± 3.7	27.1 ± 8.1 **
21–30 weeks	24	14.2 ± 4.1	23.2 ± 7.0 **
31–40 weeks	30	12.3 ± 2.7	22.7 ± 5.8 **

Significance (by paired t test) of difference between Amerlex kit and Liquisol kit
** $p < 0.001$

Amerlex kit. Remarkably, the FT_4 levels in late pregnancy (21 – 30 weeks and 31 – 40 weeks), when the serum thyroxine binding globulin level increases, were still higher than those of non-pregnant subjects (tab. 1), in contrast to the Amerlex FT_4 kit which showed lower levels than normal. As partly reported previously (7), the values obtained with the Liquisol kit gave erroneously positive results in patients undergoing triiodothyronine treatment after total thyroidectomy (theoretically zero), or with severe primary hypothyroidism (tab. 2, 3). In contrast, the values obtained with the Amerlex kit were always zero.

Tab. 2. Concentrations of circulating thyroid hormones, triiodthyronine (T_3), thyroxine (T_4), T_3 uptake (T_3U), thyroxine binding globulin (TBG) and thyrotropin (TSH) in patients with triiodothyronine treatment after total thyroidectomy.

Case No.	Age	Sex	T_3 nmol/l	T_4 nmol/l	TSH mU/l	T_3U %	TBG mg/l	FT_4 Liquisol kit pmol/l	FT_4 Amerlex kit pmol/l
1	46	♂	7.44	<12.9	<0.1	23.8	26.2	5.29	n.d.*
2	33	♀	2.96	<12.9	4.18	24.6	18.8	3.74	n.d.
3	61	♀	4.25	<12.9	2.22	20.4	26.4	3.10	n.d.
4	50	♂	4.37	<12.9	1.42	32.7	17.8	2.71	n.d.
5	49	♂	1.97	<12.9	3.14	27.4	18.0	3.48	n.d.
6	32	♂	4.40	<12.9	1.52	27.3	17.0	5.55	n.d.
7	45	♀	3.36	<12.9	2.28	20.8	26.5	3.48	n.d.
8	49	♀	4.20	<12.9	1.32	24.5	21.3	3.48	n.d.
9	60	♀	5.59	<12.9	0.25	23.6	20.4	2.97	n.d.
10	32	♂	4.74	<12.9	1.33	28.0	16.7	3.48	n.d.
11	25	♀	5.22	<12.9	1.22	22.1	20.9	3.35	n.d.
12	43	♀	3.42	<12.9	1.44	24.6	20.2	3.23	n.d.
13	31	♂	6.39	<12.9	<0.1	24.5	20.0	2.97	n.d.
14	22	♀	2.43	<12.9	4.23	25.3	20.1	2.97	n.d.
15	58	♀	2.88	<12.9	2.51	25.3	21.1	3.48	n.d.
16	49	♂	2.45	<12.9	3.82	18.7	32.4	3.48	n.d.
17	32	♂	1.96	<12.9	3.70	28.8	16.3	3.74	n.d.
Mean ± SD			4.00 ± 1.56	<12.9	2.31 ± 1.24	24.9 ± 3.4	20.6 ± 4.2	3.43 ± 60	n.d.
Normal range			1.23 – 2.93	59 – 142	0.25 – 6.00	24 – 37	12 – 30	10.3 – 31.0	9.0 – 27.1

* n.d. = not detectable
The sensitivity of FT_4 determination with the Amerlex kit is 1.94 pmol/l, and the sensitivity of the thyroxine determination is 12.9 nmol/l.

Tab. 3. Concentrations of circulating thyroid hormones, triiodothyronine (T_3), thyroxine (T_4), T_3 uptake (T_3U), thyroxine binding globulin (TBG) and thyrotropin (TSH) in patients with severe primary hypothyroidism (thyroxine less than 12.9 nmol/l).

Case No.	Age	Sex	T_3 nmol/l	T_4 nmol/l	TSH mU/l	T_3U %	TBG mg/l	FT_4 Liquisol kit pmol/l	FT_4 Amerlex kit pmol/l
1	63	♂	0.57	<12.9	120	24.6	27.0	5.29	n.d.*
2	72	♀	1.46	<12.9	104	23.1	31.0	5.81	n.d.
3	31	♀	0.51	<12.9	240 <	26.2	24.8	5.29	n.d.
4	32	♂	0.35	<12.9	240 <	25.6	18.6	3.48	n.d.
5	51	♀	0.52	<12.9	78	20.0	30.4	4.13	n.d.
6	49	♀	0.43	<12.9	28	21.8	28.0	4.77	n.d.
7	49	♀	0.75	<12.9	81	19.8	34.9	4.13	n.d.
8	53	♀	0.72	<12.9	96	24.2	28.1	5.29	n.d.
9	43	♂	0.86	<12.9	240 <	20.4	30.2	5.16	n.d.
10	45	♀	0.68	<12.9	240	23.0	25.2	4.90	n.d.
11	60	♀	0.42	<12.9	171	29.4	23.5	4.52	n.d.
12	42	♀	0.88	<12.9	125	21.8	23.9	4.77	n.d.
13	50	♀	1.02	<12.9	79	20.3	35.9	4.90	n.d.
14	42	♀	0.77	<12.9	101	33.6	25.2	3.87	n.d.
15	42	♀	0.52	<12.9	99	21.4	24.0	6.71	n.d.
16	45	♂	1.43	<12.9	70	26.7	20.8	5.55	n.d.
Mean ± SD			0.74 ± 0.33	<12.9	132 ± 71	23.9 ± 3.8	27.0 ± 4.7	4.91 ± 0.80	n.d.
Normal range			1.23 – 2.93	59 – 142	0.25 – 6.00	24 – 37	12 – 30	10.3 – 31.0	9.6 – 27.1

* n.d. = not detectable
The sensitivity of FT_4 determination with the Amerlex kit is 1.94 pmol/l, and the sensitivity of the thyroxine determination is 12.9 nmol/l.

Discussion

Numerous commercial methods are now available for the measurement of FT_4. Some claim that an accurate, precise assay of FT_4 provides the single best assessment of thyroid function (10, 11). Unfortunately, as with all clinical laboratory methods, FT_4 methods suffer from interferences. The most common is the effect of abnormal albumin concentrations (12, 13) or non-esterified fatty acids (14, 15). Since each FT_4 method behaves uniquely, each should be independently viewed with respect to interference. Common interferences (albumin effect, non-esterified fatty acid effect) have been thoroughly studied, but the effect of unsaturated thyroxine binding globulin has not yet been investigated, although it has been suspected that the performance of DPC kits (Diagnostic Products Corp., USA) is dependent on the concentration of thyroxine binding globulin in serum (16).

As shown in figure 1, the addition increasing amounts of purified thyroxine binding globulin to a test tube containing microcapsule suspension strongly affected the accuracy of the results. We found that in the presence of rather high amounts of thyroxine binding globulin, [^{125}I]thyroxine leaked through the membrane into the medium, thereby giving a falsely elevated free thyroxine result. Our finding indicates that further improvements in the microcapsule membrane are necessary for successful FT_4 determination.

So far, little attention has been given to evaluation of RIA methods for FT_4 in patients with unsaturated thyroxine binding globulin. The values obtained using the Liquisol kit were significantly higher than the expected values in patients with untreated primary hypothyroidism with negligible thyroxine (tabs. 2, 3). *Melmed* et al. (3) also reported erroneously high FT_4 levels in patients with primary hypothyroidism using the Liquisol kit. However, at that time, they did not investigate the reason for these high levels.

Theoretically, FT_4 levels in patients receiving triiodothyronine treatment after total thyroidectomy should be nil. The reason for these false positive values may lie in a methodological problem of the Liquisol kit, which relies on FT_4 moving through the microcapsule membrane. The possibility that the Liquisol kit was not properly calibrated to zero, was disproved, since it was shown that the zero serum of the Liquisol FT_4 kit was undetectable by Amerlex FT_4 radioimmunoassay.

In 1983, we put forward the hypothesis that the artificially high results obtained with the Liquisol kit are due to the leakage of ^{125}I from the microcapsule (7). This hypothesis has been validated by the experiment described in this paper. To avoid erroneous interpretations, we would therefore advise caution, especially when the results obtained with the Liquisol kit are used as a test for patients with primary hypothyroidism, since in these cases the thyroxine binding globulin concentration is usually increased (17).

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References

1. Hashimoto, T. (1986) Tests of thyroid function. Labmedica *3*, 13–20.
2. Wang, Y. S., Hershman, J. M. & Pekary, A. E. (1985) Improved ultrafiltration method for simultaneous measurement of free thyroxin and free triiodothyronine in serum. Clin. Chem. *31*, 517–522.
3. Melmed, S., Geola, F. L., Reed, A. W., Pekary, A. E., Park, J. & Hershman, J. M. (1982) A comparison of methods for assessing thyroid function in nonthyroidal illness. J. Clin. Endocrinol. Metab. *54*, 300–306.
4. Wilkins, T. A., Midgley, J. E. M. & Barron, N. (1985) Comprehensive study of a thyroxin-analog-based assay for free thyroxin ("Amerlex FT_4"). Clin. Chem. *31*, 1644–1653.
5. Wilke, T. J. (1986) Estimation of free thyroid hormone concentrations in the clinical laboratory. Clin. Chem. *32*, 585–592.
6. Mizuta, H., Miyai, K., Ichihara, K., Amino, N., Harada, T., Nose, O. & Tanizawa, O. (1982) Radioimmunoassay of "free thyroxin" in dried blood spots on filter paper – preliminary observations on the effective differentiation of subjects with congenital hypothyroidism from those with subnormal thyroxin-binding globulin and normal subjects. Clin. Chem. *28*, 505–508.
7. Hashimoto, T., Okada, T., Nagahara, M. & Matsubara, F. (1983) The effect of thyroxine (T_4) unattached thyroxine binding globulin (TBG) on free T_4 determination. In: *Current Problems in Thyroid Research* (Torizuka, N., Nagataki, S. & Miyai, K., eds.) pp. 279–282, Excerpta Medica, Amsterdam.
8. Miyai, K., Ito, M. & Hata, N. (1982) Enzyme immunoassay of thyroxin-binding globulin. Clin. Chem. *28*, 2408–2411.

9. Pensky, J. & Marshall, J. S. (1969) Studies on thyroxine-binding globulin (TGB): Separation from human serum by affinity chromatography. Arch. Biochem. Biophys. *135*, 304–310.
10. Kunst, A., Seidenschwarz, E., Burk, H., Schauer, S., Haug, H., Lehmann, P. & Ehrhardt, V. (1988) New one-step enzyme immunoassay for free thyroxin. Clin. Chem. *34*, 1830–1833.
11. Skiest, D., Braverman, L. E. & Emerson, C. H. (1986) Concentration of free thyroxin in serum of a patient with euthyroid hyperthyroxinemia secondary to increased thyroxin-binding prealbumin: results by various methods compared. Clin. Chem. *32*, 687–689.
12. Csako, G., Zweig, M. H., Benson, C. & Ruddel, M. (1986) On the albumin dependence of measurements of free thyroxin. 1. Technical performance of seven methods. Clin. Chem. *32*, 108–115.
13. Amino, N., Nishi, K., Nakatani, K., Mizuta, H., Ichihara, K., Tanizawa, O. & Miyai, K. (1983) Effect of albumin concentration on the assay of serum free thyroxin by equilibrium radioimmunoassay with labeled thyroxin analog (Amerlex Free T_4). Clin. Chem. *29*, 321–325.
14. Mardell, R. & Gamben, T. R. (1982) Artifactual reduction in circulating free thyroxine concentration by radioimmunoassays. Lancet *i*, 973–974.
15. Ekins, R. P. (1984) Free hormones in blood: the concept and the measurement. J. Clin. Immunoassay *7*, 163–180.
16. Tikanoja, S. & Liewendahl, K. (1988) Effect of thyroid hormone-binding proteins and fatty acids on modified analog assays of FT_4 and FT_3 in serum. Clin. Chem. *34*, 1361–1362.
17. Hesh, R. D., Gatz, J., McIntosh, C. H. S., Janzen, J. & Hehrmann, R. (1976) Radioimmunoassay of thyroxine-binding globulin in human plasma. Clin. Chim. Acta *70*, 33–42.

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