

## Methodological aspects for free hormone estimation using microencapsulated antibody method—The effects of hormone binding protein on permeability of microcapsule membrane

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The effect of T<sub>4</sub> non-carrying thyroxine binding globulin (TBG) on free thyroxine determination using the microencapsulated antibody method was studied, to investigate the precise reliability of the membrane and to find possible applications for estimating other free steroid hormones. When increased amounts of purified TBG were added to a test tube containing microcapsule suspension, it affected the accuracy of the results. We found that with higher amounts, <sup>125</sup>I-T<sub>4</sub> leaked through the membrane into the medium, thereby giving a falsely increased free T<sub>4</sub> result. Our finding indicates that further improvements in the microcapsule membrane are necessary; or alternatively, it may also be possible to balance the binding affinity inside and outside the membrane by adding a suitable amount of carrier protein, to the contents of the capsule, so that both successful FT<sub>4</sub> determination and other free steroid hormone assays may be undertaken.

**Key words:** Free thyroxine, Microencapsulated antibody method, Free hormones

### INTRODUCTION

THE ESTIMATION of free hormone levels in the blood, when the hormone has its binding protein, is remarkably accurate when compared to the total hormone estimation. In fact, free thyroxine (FT<sub>4</sub>) determination is now a routine procedure for the evaluation of thyroid function.<sup>1-4</sup> In a report by Chopra and Tulchinsky<sup>5</sup> on the pathogenesis of gynecomastia in hyperthyroid men, the estimation of free estrogen and free testosterone was the most important tool in clinical investigation. If a simple and appropriate method, similar to FT<sub>4</sub> determination in the thyroid disease, were available, it would be very beneficial in determining other endocrine disorders caused by free hormone imbalance.

Buehler<sup>6</sup> has reported using the microencapsulated antibody method in determining FT<sub>4</sub> with radioimmunoassay (RIA) and showed it to be applicable for assaying free cortisol and free testosterone. This

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unique microencapsulated antibody method seemed to be attractive, practicable and promising for several free hormone estimations, but it did not become a commercial success. In order to make further improvements in technology, at first we considered it important to examine the reliability of the microcapsule membrane.

In this paper we describe our assessment of some methodological problems in the microencapsulated antibody method used in the FT<sub>4</sub> RIA kit, which will have important significance when a successful assay method for free testosterone or free cortisol is developed in the future.

### MATERIALS AND METHODS

#### Experimental procedures:

##### *Preparation of TBG*

Highly purified TBG was prepared from pooled human serum by affinity chromatography as previously described.<sup>7</sup> The technique employed was similar to the method described by Pensky and Marshall.<sup>8</sup> <sup>125</sup>I thyroxine was added to the purified TBG and the mixture was subjected to disc electro-

phoresis. A single protein band coincided with the radioactivity. The TBG did not contain any  $T_4$  after purification. The purified TBG zero serum contains a concentration of 4.0 g/dl albumin, 10.1 mg/dl pre-albumin and 0.54 mmol/l of nonesterified fatty acids (NEFA).

#### Assay procedures

The assay for  $FT_4$  using the microcapsule method (Liquisol  $FT_4$  kit Damon Diagnostics, USA) was performed in accordance with the manufacturer's instructions. Briefly, twenty-five  $\mu$ l of saline or purified TBG (0, 13, 52  $\mu$ g/ml) was added to test tubes containing 500  $\mu$ l of microcapsule suspension which contained anti- $T_4$  combined with  $^{125}I$ - $T_4$ . Twenty-five  $\mu$ l of standard solution (0, 0.15, 0.38, 1.10, 2.00, 3.10 and 5.00 ng/dl) was pipetted into each tube and the tubes were vortexed for 4 seconds. After the tubes were incubated in a water bath at 37°C for one hour, they were vortexed for 4 seconds, and then incubated for an additional one hour. After that, one ml of washing solution (polyethyleneimine) was added to each tube followed by 4 seconds vortex. The tubes were incubated at room temperature for 20 minutes, and subsequently centrifuged at 1,400 g for 10 minutes. After decanting the supernatant, the sedimenting microcapsules were counted in a gamma counter for 1 minute. The intra- and inter-assay coefficients of variation were 7.2–8.5% between 0.40 and 4.50 ng/dl and 14.7–15.9% between 1.00 and 3.00 ng/dl respectively.

$FT_4$  was also measured with an Amerlex kit (Amersham, U.K.);  $T_3$ ,  $T_4$ , TSH, thyroglobulin (TG), TBG with an EIKEN kit (EIKEN ICL, Japan), and  $T_3$  RSU with a Dainabot kit (Dainabot Co. Japan). The accuracy of these kits and the analytical procedures for measurement of these hormones have previously been described in detail.<sup>9,10</sup> All samples were measured simultaneously in order to avoid inter-assay variations.

#### Patient materials

Seventeen patients receiving  $T_3$  replacement therapy after total thyroidectomy ( $T_4$  less than 1  $\mu$ g/dl) were studied. Sera were aliquoted and stored at -20°C until the assay was undertaken.

#### Statistical Analysis

The results were expressed as the mean  $\pm$  standard deviation (SD). The significance of differences between mean values was evaluated by the paired t-test.

## RESULTS AND DISCUSSION

Figure 1. illustrates the effect of varying doses of purified TBG from 0 to 52  $\mu$ g/ml on a Liquisol  $FT_4$

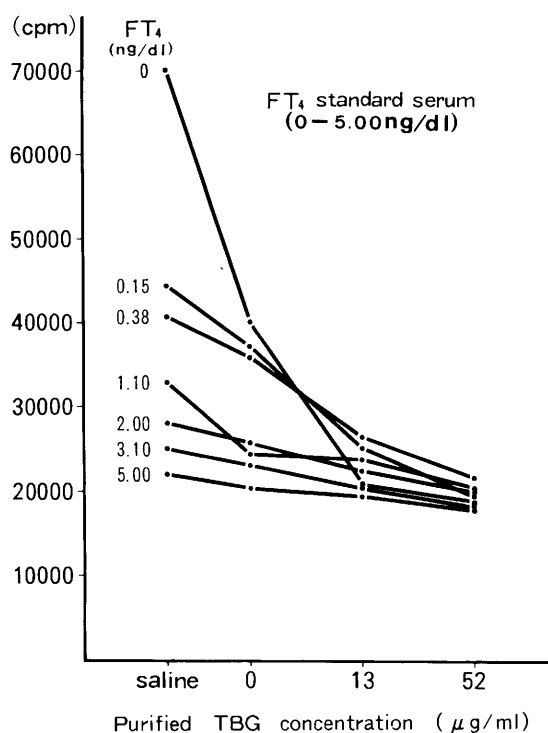


Fig. 1 Effect of different concentrations of TBG on  $FT_4$  determination (Liquisol  $FT_4$  kit).

Note: Because of the leakage of  $^{125}I$ - $T_4$  from the microcapsule, the standard curve can no longer be distinguished at a concentration above 13  $\mu$ g/ml of the purified TBG.

kit. The results show that the  $FT_4$  values determined by this method are strongly influenced by the concentration of TBG in the samples: when the added purified TBG concentration outside of the capsules was high,  $^{125}I$ - $T_4$  was extracted from the microcapsules. Because of this leakage, the  $FT_4$  standard curve can no longer be distinguished at a concentration above 13  $\mu$ g/ml of the purified TBG. Figure 2. is a schematic diagram of the effect of uncomplexed TBG on  $FT_4$  determination using the microencapsulated antibody method. The difference between the  $^{125}I$  radioactivity in the microcapsules dissolved in saline and those dissolved in the purified zero TBG solution might be mostly due to the fact that the TBG solution contains human albumin and pre-albumin, which affected the leakage of the encapsulated  $^{125}I$ - $T_4$ .<sup>11</sup> Each standard solution in the Liquisol  $FT_4$  kit contains approximately 3.0 g/dl of human albumin and 17.8  $\mu$ g/dl of TBG. The reason for the distinctive and exceptional decline in Liquisol  $FT_4$  zero serum is inexplicable. Whatever the reason, the difference between the results in the saline and the purified zero TBG solution indicate, that saline should not be used for dilution.

As we<sup>10</sup> and Melmed et al.<sup>12</sup> reported previously, the values obtained with the Liquisol kit were signi-

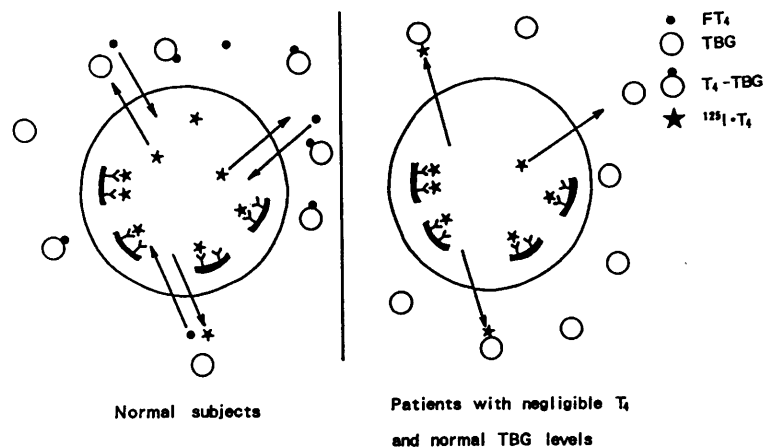


Fig. 2 A schematic diagram of the effect of uncomplexed TBG on FT<sub>4</sub> determination (Liquisol FT<sub>4</sub> kit).

Table 1 Concentrations of circulating thyroid hormones, TBG and TSH in patients with T<sub>3</sub> treatment after total thyroidectomy\*

Case No.	Age & Sex	T <sub>3</sub> ng/dl	T <sub>4</sub> μg/dl	F-T <sub>4</sub> Liquisol kit ng/dl	F-T <sub>4</sub> Amerlex kit ng/dl	TBG μg/ml	TSH μU/ml
1	64 M	283	<1	0.46	n.d.	24.3	<0.1
2	32 M	192	<1	0.24	n.d.	20.7	0.51
3	46 M	276	<1	0.25	n.d.	25.4	<0.1
4	48 M	284	<1	0.23	n.d.	18.8	<0.1
5	51 M	230	<1	0.30	n.d.	19.0	1.10
6	32 M	286	<1	0.43	n.d.	17.0	<0.1
7	45 F	218	<1	0.27	n.d.	25.5	0.45
8	49 F	273	<1	0.27	n.d.	21.3	0.20
9	60 F	263	<1	0.23	n.d.	20.4	0.18
10	32 M	308	<1	0.27	n.d.	18.7	<0.1
11	25 F	239	<1	0.26	n.d.	20.9	0.15
12	43 F	222	<1	0.25	n.d.	20.2	0.82
13	31 M	215	<1	0.23	n.d.	20.0	1.15
14	28 F	255	<1	0.28	n.d.	20.1	1.06
15	58 F	187	<1	0.27	n.d.	21.1	2.50
16	50 F	260	<1	0.30	n.d.	28.4	<0.1
17	30 F	235	<1	0.27	n.d.	18.5	<0.1
Mean±SD		249±30	<1	0.29±0.06	n.d.	21.2±4.2	
Normal values		141±23	6.5±1.4	1.9±0.4	1.2±0.3	21.4±4.3	0.25–6.00

n.d.: not detectable; The sensitivity of the FT<sub>4</sub> Amerlex kit is 0.15 ng/dl.

\*Note: In these patients, TBG is saturated only by T<sub>3</sub>, and the "excess" (non T<sub>4</sub> bound) TBG causes the leakage of the encapsulated <sup>125</sup>I-T<sub>4</sub> through the microcapsule membrane, thereby giving a falsely increased FT<sub>4</sub> result with the Liquisol kit.

ificantly higher than those expected in patients with primary hypothyroidism. In this study, as shown in Table 1., this kit gave erroneously higher concentrations, in contrast with the Amerlex kit, in patients undergoing T<sub>3</sub> treatment after total thyroidectomy.

Previously, the microencapsulated antibody method used in FT<sub>4</sub> RIA was reported to be applicable for the assay of free fractions of cortisol or testosterone by Buehler.<sup>6</sup> However, plasma free cortisol or tes-

tosterone determined by this principle could also be affected by the binding protein for each hormone in a similar manner. It has been reported by Key and Moore<sup>13</sup> and Slaats et al.<sup>14</sup> that sex-hormone binding globulin (SHBG) has interfered with estradiol and testosterone estimation in a non-extraction RIA method.

Although many investigators have reported various methods for measuring free aldosterone,<sup>15</sup> free corti-

sol,<sup>16</sup> free deoxycorticosterone,<sup>17</sup> free estrogens,<sup>18</sup> and free testosterone,<sup>19</sup> the methods for their assays are complicated, time consuming and not practical in clinical use. Despite the methodological problems which are not completely solved yet, the micro-encapsulated dialysis method appears to have the potential to be a sensitive, flexible and practicable method for the determination of various free hormones. In addition, this method is principally a miniature dialysis system and there is a consensus that free hormones determined by equilibrium dialysis should be a reference standard against which other measurements are compared.<sup>20</sup>

In conclusion, further improvements of the microcapsule membrane and/or addition of carrier protein to the capsule, to balance the binding affinity inside and outside the membrane, are necessary for the determination of FT<sub>4</sub> and other free steroid hormones. If further improvements in this area of technology are able to be made, we will have an easier and more precise method of estimating all free hormones.

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