Annals of Nuclear Medicine Vol. 1, No. 1, 15–22, 1987

Measurements of serum-free thyroid hormone concentrations by ultrafiltration — a comparison with equilibrium dialysis and mathematical calculation —

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An ultrafiltration method (UF) for measuring free thyroxine (FT_4) and free triiodothyronine (FT_3) using the Diaflow YM membrane (Centricon-10) is described. The results are compared with those by equilibrium dialysis (ED) and also by mathematical calculations derived from T_4, T_3, and binding protein concentrations. The precision with the UF method was excellent. The normal ranges of FT_4 and FT_3 by the three methods are all comparable. There was a high degree of correlation of FT_4 or FT_3 results by UF with those by ED and by calculation ($r=0.940$–0.974, $n=161$, $P<0.001$). FT_4 and FT_3 by all methods agreed well for hyperthyroidism, hypothyroidism, and for patients with low T_4-binding globulin. The mean FT_3 in pregnancy was lower than the normal value for all methods, and FT_4 concentrations by UF and calculation also decreased in late pregnancy. The mean FT_4 by UF and ED in low T_3 syndrome were significantly higher than in the normal controls, while the calculated FT_4 was lower. The FT_3 in low T_3 syndrome distributed normal to subnormal in all methods. These results indicate that a) the UF method is a reliable reference method for measuring FT_4 and FT_3 concentrations; b) the UF results agree well with those by ED and also with theoretically derived values in subjects with thyroid diseases and TBG abnormalities; c) for patients with low T_3 syndrome, the FT_4 results obtained by UF and ED are similarly discrepant from the calculated results, implying the existence of binding inhibitor(s) which affect both UF and ED measurements.

Key words: Free thyroid hormones, Ultrafiltration, Equilibrium dialysis, Calculation, Diagnostic aid, Thyroid function

INTRODUCTION

CIRCULATING THYROID HORMONES, thyroxine (T_4) and triiodothyronine (T_3), are bound to the binding proteins, T_4-binding globulin (TBG), T_4-binding prealbumin (TBPA), and albumin, and only very small fractions of T_4 and T_3 are in the unbound (free) form in human serum.[1] Because free T_4(FT_4) and free T_3(FT_3) are generally not affected by variations in T_4-binding proteins,[2,3] FT_4 and FT_3 concentrations reflect the thyroid function more closely than the total concentrations of T_4 and T_3.

The most widely used method for estimation of FT_4 and FT_3 is equilibrium dialysis (ED),[4,5] but this method is time-consuming and not convenient for clinical use. To overcome this, a number of radioimmunoassays (RIA) based on different principles have been developed for estimates of FT_4 and FT_3, but results with these may be influenced by changes in serum proteins and by the effect of nonthyroidal illnesses (NTI).[6,7]

Ultrafiltration techniques have been developed in recent years for use with dialysis tubing or micro-

Received April 28, 1987; revision accepted April 28, 1987.

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partition systems[8–13] and this technique is an alternative to ED and RIA. In the present study, we evaluate the ultrafiltration technique using Amicon YM Diaflow membrane for FT_4 and FT_3 measurements and compare the results with those by ED and mathematical calculations.

MATERIALS AND METHODS

Patients

Six groups of patients were studied: (I) 45 normal subjects who visited the hospital for routine health examinations, ages 38–68 years; (II) 17 hyperthyroid patients with supranormal T_4 and T_3; (III) 10 hypothyroid patients with subnormal T_4 and (or) T_3 and supranormal thyrotropin; (IV) 5 patients with low TBG; (V) 40 pregnant women; (VI) 44 patients with NTI, including 9 patients with acute hapatitis associated with supranormal TBG, and 35 patients with low T_3 syndrome. The etiological diagnoses of the low T_3 syndrome include malignant neoplasma (22 cases), cerebral vascular disorders (four cases), rheumatoid arthritis (three cases), renal failure (two cases), cardiac failure (two cases), pulmonary failure (one case), and hepatic failure (one case). None of these patients were treated with heparin or aspirin. All serum samples were stored at −20°C until assayed.

Reagents and Apparatus

Tracer. T_4 labeled with ^{125}I (T_4 tracer) and T_3 labeled with ^{125}I (T_3 tracer), both with a listed specific radioactivity of more than 1.2 mCi/μg dissolved in 75% ethanol, were obtained every four weeks from Amersham International plc. On receipt, the tracers were evaporated under nitrogen at 37°C, and human serum albumin (Sigma Chemical Company, T_4 and T_3 concentrations in the albumin were 0.08 μg/g, and 5.5 ng/g, respectively by RIA) in 0.063 M phosphate buffer (pH=7.4) in 0.05% saline was added to yield final concentrations of 1.0 to 1.2% of albumin and incubated for 20 min at 37°C in darkness. The mixture was dialyzed overnight at 4°C against 2.0 L of phosphate buffer to remove labeled contaminants of the tracers. This purification procedure was performed every two weeks. The paper chromatographic analysis (butanol: acetic acid: water, 78: 5: 17 v/v) showed that the dialyzed specimens of T_4 and T_3 tracers contained less than 1.0 % iodide. The tracer concentration for the assay was approximately 20 μCi/m*l*, or 16.7 μg/L for both T_4 and T_3.

Apparatus. For the ultrafiltration, a Centricon centrifugal microconcentrator (Centricon-10, Amicon Corp., Lexington, Mass, USA) with a Diaflow YM-10 membrane and membrane support base, an O-ring, and a filtercup was used. This membrane has a relative molecular mass (Mr) cut-off of 10,000 daltons.

Procedures

Ultrafiltration. We added 50 μl of T_4 tracer or 25 μl of T_3 tracer to 100 μl of serum diluted with 650 μl (for FT_4 assay) or 675 μl (for FT_3 assay) of 0.15 M phosphate buffer (pH=7.4) in 0.05% saline. After mixing, the diluted sera were kept in the test tubes for 10 min at room temperature. A 700 μl portion of each serum sample was transferred to the Centricon-10 device, and incubated for a further 20 min at 37°C. It was centrifuged at 1,000×g for 40 min at 37°C. Operation instructions recommend the use of a fixed angle rotor for centrifugation, however a conventional swinging-bucket rotor suitable for obtaining a sufficient volume of filtrate was used. A 300 μl portion of filtrate was added to 1.7 m*l* of phosphate buffer and 1 m*l* of the carrier T_4 and T_3 solution. The radioactive T_4 and T_3 were separated by magnesium chloride precipitation techniques as described elsewhere.[5,14] We determined the total radioactivity by counting a 24 μl aliquot of the original diluted serum samples. The recovery of T_4 and T_3 by $MgCl_2$ precipitation were 95% and 83%, respectively. The free fractions of iodothyronines were calculated as follows:

$$\frac{\text{CPM of labeled precipitate in 300 } \mu l \text{ of filtrate}}{\text{CPM of labeled } T_4 \text{ or } T_3 \text{ in 25 } \mu l \text{ of serum} \times 12 \times 8} \times 100$$

where the 8 in the denominator is the dilution factor. For the FT_3 assay, the results were corrected for the 83% yield during the $MgCl_2$ precipitation. FT_4 and FT_3 concentrations were expressed as the products of total T_4 (or T_3) and $\%FT_4$ (or $\%FT_3$). By this procedure, 20 single samples can be assayed in less than three hours.

Equilibrium Dialysis. FT_4 and FT_3 in the 80-fold diluted sera were also measured by equilibrium dialysis with the tracer method and $MgCl_2$ precipitation techniques as described elsewhere[5,14].

Mathematical Calculation. The calculation of free thyroid hormone concentrations was made from the following equation, according to Lécureuil et al[15]:

$$\begin{aligned} &F^2K_{TBG}[1+C_{TBPA}(K_{aTBPA}+K_{bTBPA}) \\ &+C_{Alb}(K_{aAlb}+nK_{bAlb})]+F[C_{TBG}K_{TBG} \\ &+C_{TBPA}(K_{aTBPA}+K_{bTBPA}) \\ &+C_{Alb}(K_{aAlb}+nK_{bAlb})+1-K_{TBG}T_{4(3)}] \\ &-T_{4(3)}=0 \end{aligned}$$

where, F is the molar concentration of FT_4 or FT_3, C_{TBG}, C_{TBPA}, C_{Alb} are the molar concentrations of

Table 1 Binding constants used in the calculation

Proteins	No. of sites	K_{assoc} (L/mol)			
		T_4	Ref. No	T_3	Ref. No
TBG		2.3×10^{10}	(16)	1.15×10^{9}	(16)
TBPA	1	1.5×10^{8}	(17)§	5.0×10^{6}	(17)
	2	1.5×10^{6}	(17)	2.0×10^{5}	(18)
Albumin	1	1.4×10^{6}	(19)	1.0×10^{5}	(19)
	2–6	8.0×10^{4}	(20)	7.0×10^{3}	(21)

§Within range quoted by various authors listed in Table 1 of paper from Princé and Ramsden.[17]

TBG, TBPA and albumin, and n is the number of binding sites of albumin. We selected values for the binding constants (K) that were within the wide ranges quoted by different authors (Table 1)[16–21]. The program was written for an M-20 (Olivetti, Japan) personal computer.

Total T_4, T_3, TBG, TBPA and albumin. Total T_4, T_3, and TBG were measured by RIA.[14,22] Serum TBPA and albumin were measured by immunoturbidimetry.[23] Albumin concentration in the ultrafiltrate was measured by sensitive RIA for urine analysis (Albumin RIA kit, Diagnostic Products Corporation, USA).

Precision of Assay

Interassay CV (n=11) were: T_4 1.8%; T_3 4.4%; TBG 7.9%; TBPA 4.7%; albumin 6.9%; %FT_4 by ED 11.0%; %FT_3 by ED 12.5% for normal control sera.

Statistical Analysis

Analysis was by least-square regression, and groups were compared by Student's *t*-test.

RESULTS

In preliminary experiments, we used the albumin RIA kit for urine analysis (approximate sensitivity 0.1 mg/L) to detect protein leakage into the ultrafiltrates. Leakage was 0.00158±0.00036% (mean± S.D.) (n=4) albumin from normal sera during ultrafiltration at 37°C for 40 min. This suggests that the protein leakage through the membrane does not cause appreciable error in the FT_4 and FT_3 measurements.

During centrifugation at 1,000×g, progressive increase in filtrate volume was seen from 10 through 60 min. The %FT_4 and %FT_3 for each centrifugation period were measured with 8-fold diluted sera, and all values were nearly constant (Fig. 1).

The binding of thyroid hormones to the Centricon-10 devices was examined. An 800 *μl* volume of 8-fold diluted sera enriched with tracers was centrifuged at

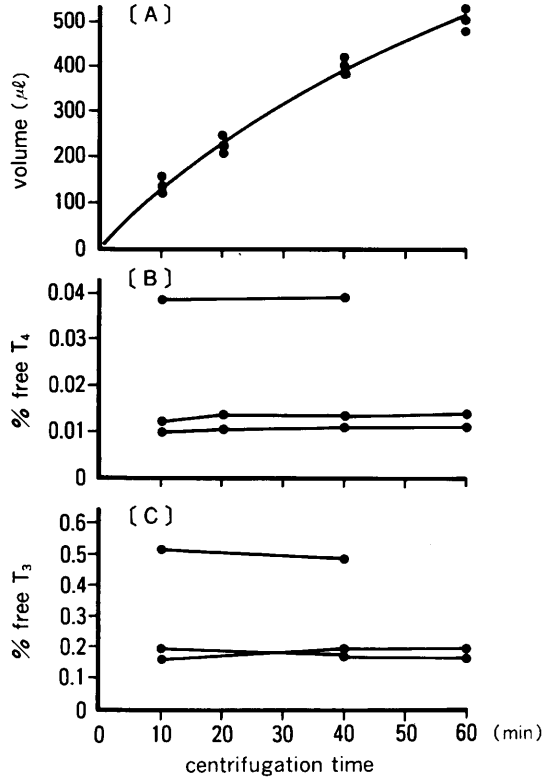

Fig. 1 Effect of centrifugation time on filtration volume (A), %FT_4 (B), and %FT_3 (C) in ultrafiltration method for FT_4 and FT_3 measurements. Three sera were tested for %FT_4 and %FT_3 measurements.

37°C for 40 min at 1,000×g, and radioactivity bound to the device was counted after three times washing with water. The binding of T_4 was 0.18±0.014% (n=3) and for T_3 it was 0.39±0.09% (n=3) of total.

The effect of temperature during centrifugation on %FT_4 and %FT_3 was also examined, and there were progressive increases in both fractions from 4°C to 37°C.

The deiodination of T_4 and T_3 tracers during the 20 min incubation at 37°C was examined by paper chromatographic analysis using 8-fold diluted sera. With T_4 tracer containing 0.5% iodide, or T_3 containing 0.2% iodide added to sera, the iodide contamination of T_4 tracer after 20 min of incubation was 0.63±0.12% (n=5) and for T_3 tracer, 0.48± 0.1% (n=5).

The effect of serum dilution of %FT_4 and %FT_3 is shown (Fig. 2). The dilution of sera from normal subjects, hyperthyroidism and pregnancy patients showed an initial decline (approximately 30%) up to 8-fold dilution. Further dilution had little effect on both %FT_4 and %FT_3. In the serum from low T_3 syndrome subjects, there was a progressive decline of %FT_4 (48.3%) and %FT_3 (35.3%) at 8-fold dilution and they declined further thereafter.

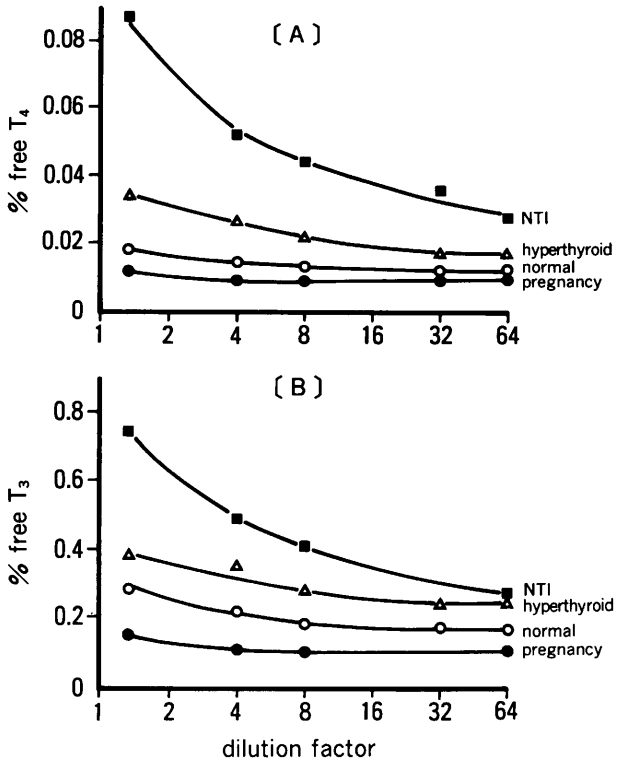

Fig. 2 Effect of serum dilution on $\%FT_4$ (A) and $\%FT_3$ (B) by ultrafiltration method in sera from normal subjects, hyperthyroid patients, pregnant women, and patients with low T_3 syndrome.

The intra-assay precision (CV) of the UF method was 2.2% for $\%FT_4$ (n=5), and 3.1% for $\%FT_3$ (n=5), and the inter-assay CV was 6.8% for $\%FT_4$ (n=10) and 12.6% for $\%FT_3$ (n=10) for samples in the normal range.

The mean values (±S.D.) for measured and calculated free thyroid hormones, together with the values for the various parameters we used in our computation, are shown in Table 2, and the individual values in low TBG, pregnancy, and NTI are also shown in Fig. 3 and Fig. 4. The normal ranges of FT_4 were 0.95×10^{-11} to 2.4×10^{-11} mol/L for UF, 10^{-11}—2.1×10^{-11} mol/L for ED, and 0.95×10^{-11}—2.2×10^{-11} mol/L for calculated FT_4. The normal ranges of FT_3 concentrations were 2.1×10^{-12}—4.6×10^{-12} mol/L for UF, 2.1×10^{-12}—5.0×10^{-12} mol/L for ED, and 2.4×10^{-12}—5.0×10^{-12} mol/L for calculated FT_3. The measured and calculated normal ranges for FT_4 and FT_3 were not significantly different.

Compared with the values for normal controls, both FT_4 and FT_3 by these methods were significantly higher in hyperthyroidism and lower in hypothyroidism. Both mean values for FT_4 and FT_3 by these methods were similar to the corresponding normal controls in the low TBG group. The mean FT_4 concentrations of the pregnant women in the 2nd and 3rd trimester were significantly lower than the mean value for normal controls, whether calculated or measured by UF, but not by ED although the mean FT_4 by ED was lower than for normal controls. The FT_3 values in the pregnant women in all trimesters were significantly lower than in the normal controls with all the methods. The individual values for FT_4 and FT_3 showed that both measured and calculated FT_4 in pregnancy distributed essentially within the normal ranges, while the FT_3 values were subnormal in 5 of 39 for UF, and in 7 of 39 for ED.

The mean FT_4 values in NTI with high TBG were normal in all methods employed, but the FT_3 values in this group were significantly lower than the normal values by calculation and measured with UF but not by ED. The measured FT_4 concentration for low T_3 syndrome was significantly higher than that of normal controls, whereas the calculated value was significantly lower. Individual FT_4 also showed that the measured FT_4 values distributed from subnormal to supranormal, in contrast to the normal-to-subnormal distribution of the calculated FT_4 values in NTI. The FT_3 concentrations, both measured and calculated, distributed from normal to subnormal in NTI, and the mean values in the low T_3 syndrome were significantly lower than the corresponding normal values.

Table 3 summarizes the regression analysis of the results obtained by the three methods for FT_4 and FT_3 measurements. When all data were analyzed together, the best correlations were between UF and ED methods for FT_4 and FT_3 (r=0.974, and 0.972, respectively). The calculated FT_4 or FT_3 has a similar correlation with the UF and ED methods. When each subgroup was subjected to separate statistical analysis, there was poor correlation between UF and ED for FT_3 in normal subjects and in pregnant women, and between ED and calculations for FT_3 in normal subjects and in pregnant women. The calculated FT_4 and FT_3 values showed good correlations with both ED and UF methods for all groups analyzed.

DISCUSSION

Determination of free thyroid hormone concentrations using the Amicon ultrafiltration device with a Diaflow YM membrane offers several advantages. This method is rapid, reproducible, and technically simple. A minimal serum albumin leak will not produce appreciable errors in the measurements. The $\%FT_4$ and $\%FT_3$ values are unchanged throughout the centrifugation. In addition, binding of tracers to the device and deiodination of tracers are minimal

Table 2 Values for calculated and measured serum free thyroid hormone concentrations in various groups

	Normal (n=45)	Hyper-thyroidism (n=17)	Hypo-thyroidism (n=10)	Low TBG (n=5)	Pregnancy			Nonthyroidal illnesses with	
					First trimester (n=11)	Second trimester (n=13)	Third trimester (n=16)	High TBG (n=9)	Low T_3 (n=35)
T_4	118§	287[a]	44[a]	60[a]	144[a]	174[a]	167[a]	153[a]	82[a]
(10^{-9} mol/L)	(20)	(74)	(21)	(11)	(40)	(26)	(37)	(27)	(29)
T_3	1.89	6.31[a]	1.09[a]	1.08[a]	2.09[a]	2.52[a]	2.45[a]	2.28[a]	0.74[a]
(10^{-9} mol/L)	(0.27)	(1.45)	(0.41)	(0.09)	(0.45)	(0.35)	(0.45)	(0.46)	(0.24)
TBG	2.95	2.58[b]	3.51[b]	0.89[a]	4.20[a]	6.55[a]	6.18[a]	5.06[a]	2.57[a]
(10^{-7} mol/L)	(0.39)	(0.30)	(0.41)	(0.50)	(0.91)	(0.81)	(1.03)	(0.42)	(0.73)
TBPA	65.3	41.6[b]	63.9	62.6	57.1[b]	45.8[b]	51.6[b]	32.7[a]	35.7[a]
(10^{-7} mol/L)	(17.6)	(14.0)	(11.3)	(10.8)	(8.9)	(9.6)	(6.5)	(10.2)	(16.5)
Albumin	589	514[b]	585	557	547[b]	455[a]	421[a]	480[b]	379[a]
(10^{-6} mol/L)	(66)	(87)	(100)	(69)	(83)	(62)	(52)	(54)	(83)
FT_4 (10^{-11} mol/L)									
UF	1.40	7.58[a]	0.40[a]	1.28	1.30	1.16[b]	1.22[c]	1.47	1.73[a]
	(0.30)	(4.67)	(0.22)	(0.38)	(0.39)	(0.35)	(0.21)	(0.41)	(0.67)
ED	1.52	8.77[a]	0.46[a]	1.65	1.50	1.37	1.37	1.51	2.15[a]
	(0.27)	(6.39)	(0.28)	(0.57)	(0.56)	(0.21)	(0.34)	(0.48)	(0.99)
Calculated	1.57	7.01[a]	0.46[a]	1.50	1.51	1.29[a]	1.32[a]	1.45	1.35[b]
	(0.31)	(4.01)	(0.25)	(0.04)	(0.37)	(0.20)	(0.24)	(0.28)	(0.36)
FT_3 (10^{-12} mol/L)									
UF	3.56	22.9[a]	1.34[a]	3.75	2.98[b]	2.60[b]	2.63[b]	3.14[c]	1.99[a]
	(0.72)	(14.9)	(0.71)	(0.77)	(0.71)	(0.30)	(0.40)	(0.66)	(0.79)
ED	3.62	26.2[a]	1.57[a]	3.40	2.78[c]	2.62[a]	2.73[a]	3.35	1.98[a]
	(0.71)	(16.9)	(0.73)	(0.94)	(0.90)	(0.38)	(0.58)	(0.87)	(0.72)
Calculated	3.89	16.9[a]	2.22[a]	4.34	3.41[b]	2.91[a]	2.98[a]	3.29[a]	1.89[a]
	(0.58)	(7.1)	(0.88)	(0.83)	(0.63)	(0.21)	(0.31)	(0.63)	(0.48)

§Mean (S.D.) is shown. [a-c] significantly different from normal control by $p<0.001^a$, 0.01^b, or 0.05^c.

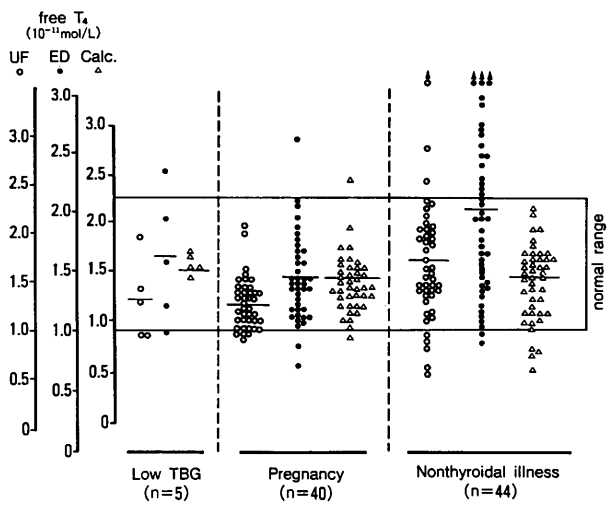

Fig. 3 Individual values for FT_4 concentrations as measured by ultrafiltration (UF), equilibrium dialysis (ED), and by mathematical calculation (calc.) in low TBG, pregnancy, and nonthyroidal illnesses. Data for pregnancy include all FT_4 values in Table 2. Arrows indicate FT_4 value above 3.5×10^{-11} mol/L in UF and ED methods.

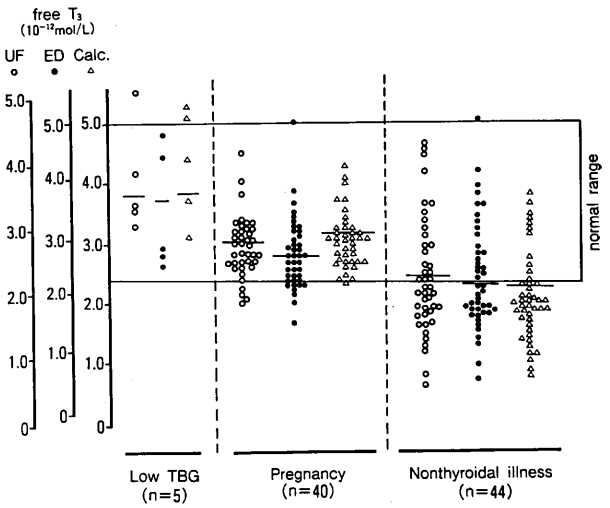

Fig. 4 Individual values for FT_3 concnetrations as measured by ultrafiltration (UF), equilibrium dialysis (ED), and mathematical calculation (calc.) in low TBG, pregnancy, and nonthyroidal illnesses. Pregnancy includes first (11), second (13), and third (16) trimester pregnant women.

Table 3 Correlations between free thyroid hormone concentrations as measured by ultrafiltration, equilibrium dialysis, and mathematical calculation

		Normal (n=45)	Hyper-thyroidism (n=17)	Hypo-thyroidism (n=10)	Pregnancy (n=40)	Nonthyroidal illnesses (n=44)	Total (n=161)
Ultrafiltration VS. Equilibrium dialysis	FT_4	0.705	0.963	0.971	0.732	0.758	0.974
	FT_3	0.378	0.933	0.830	0.473	0.737	0.972
Ultrafiltration VS. Calculation	FT_4	0.680	0.872	0.954	0.778	0.656	0.942
	FT_3	0.640	0.930	0.915	0.820	0.774	0.958
Equilibrium dialysis VS. Calculation	FT_4	0.620	0.912	0.903	0.792	0.419	0.940
	FT_3	0.471	0.899	0.878	0.420	0.800	0.949

Pregnancy includes all data listed in Table 2. The total is all the subjects in Table 2, including patients with low TBG (n=5). All values for correlation coefficients in this table are statistically significant ($p<0.01$).

during the procedures. These points must be added to the evaluations in previous reports.[11-13]

The normal ranges for FT_4 and FT_3 concentrations by the UF method in the present study were somewhat lower than previously shown with undiluted sera.[9,10] This is presumably due to the effect of serum dilution, as the data showed serum dilution to produce a lowering of free thyroid hormone fractions, as previously shown in theoretical and experimental studies.[17,24-26]

There are reports where the free thyroid hormone concentrations by the UF method were compared with the ED method,[11-13] however no reports have included a comparison with mathematical calculations. The mathematical model for the interaction between thyroid hormones and their transport proteins in sera has been proposed by several investigators.[15,17,27,28] The calculated values for FT_4 and FT_3 varied considerably, possibly because of different values for affinity constants of the binding proteins employed in the calculations.[27,28] The model and calculation method by Lécureuil et al,[15] which we used here, is simpler than that by Princé and Ramsden.[17] The affinity constants used here can still be considered acceptable, as the measured and calculated normal free thyroid hormone concentrations were quite similar. The values for both methods also correlated well with each other in all the combined data, and also in the various groups analyzed separately.

The calculated and measured free thyroid hormone concentrations agree well with each other and are predictable in hyperthyroidism, hypothyroidism, and in patients with low TBG levels. The FT_4 concentrations decrease as pregnancy progresses in the calculated values as well as in the UF measured values but not in the ED-measured FT_4. The mean FT_3 values are also lower than the normal values in pregnancy for all methods. The reason for the discrepancy with the FT_4 results in late pregnancy as measured by UF and ED is unknown, but large variation in the ED method may be involved because the interassay CV for ED is larger than that for UF. Although the reduced FT_4 and FT_3 levels in late pregnancy were established theoretically,[29] there is disagreement with previous investigations on the UF measured FT_4 and FT_3 values,[3] Our data agree with Shannon et al,[12] Weeke et al,[10] and Lee et al,[30] but not with Faber et al,[9] Sophianopoulos et al,[11] and Wang et al.[13] The FT_4 and FT_3 concentrations by the present UF method agree well with the calculated values, and it is apparent that this method may be useful in evaluating the thyroid functional status with altered TBG concentrations.

We found discrepancies between measured and calculated concentrations of FT_4 but not for FT_3 in low T_3 syndrome. The FT_4 as measured by UF and ED were both significantly higher than the normal values, while the calculated values were conversely lower. Similar discrepancies were reported by Brown-Grant et al. using a different model for FT_4 calculations and the ED method.[27] One of the reasons for this discrepancy may be due to the presence of compound(s) that interfere with hormone-protein interaction.[31] The progressive decline of FT_4 and FT_3 by serum dilution in the low T_3 syndrome are in accord with the findings by Nelson et al,[26] and Weeke et al,[10] and may be interpreted to mean that the serum from this syndrome may contain such an inhibitor(s). Chopra et al have shown that free fatty acid is a likely candidate for the inhibitor, and this inhibitor affects the T_4 binding stronger than the T_3 binding.[31] Although the role of free fatty acid has been discounted recently,[32] our agreement between calculated and measured FT_3 in the low T_3 syndrome, unlike the FT_4, may be consistent with

Chopra et al. Our data also indicate that the inhibitor may be equally effective with ED and UF systems. However, we cannot exclude other possibilities such as an alteration of binding characteristics of TBG,[33] or the presence of a different type of TBG in the sera of low T_3 syndrome.[34]

The principles of the UF method have been well established,[35] and we describe a method for measurements of FT_4 and FT_3 which is a reliable and useful reference method for evaluation of thyroid functional status in clinical and experimental studies.

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