In Vitro

Choice of Reference Sera for Measurement of Effective Thyroxine Ratio

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The measurement of combined T_3U and T_4D by the use of effective thyroxine ratio (ETR), compensated thyroxine (CT_4), or normalized thyroxine (NT_4) systems has become commonplace. All these methods rely on comparison of individual patient results to a euthyroid reference serum; however, little has been published regarding the requirements of an acceptable euthyroid reference serum. Measurement of T_3U, T_4D, and ETR using acid denaturation in sera with varying T_3U and T_4D indicates that ETR is systematically dependent on total T_4 concentration, but relatively insensitive to thyroxine binding globulin level. In selecting an appropriate euthyroid reference serum for either ETR, NT_4, or CT_4 procedures, the T_4 value must be scrutinized, whereas T_3U is of less importance. If successive reference sera are not carefully selected, the published reference ranges from normal populations could be invalid.

Various thyroid function indices have been proposed in an attempt to define a single parameter that is indicative of the circulating free thyroid hormone concentration. Empirical ratios have been proposed for two major reasons: to simplify the interpretation of results in physiological states with altered transport protein levels (*1, 2*); and to correct for the interassay variability in the testing process (*3*).

One of the more recent indices proposed to simplify thyroid function testing is the effective thyroxine ratio (ETR)™ (Mallinckrodt Chemical Works, St. Louis, MO) (*4*), which purports to provide a single quantitative laboratory parameter reflecting both the total circulating thyroxine level, and the thyroxine binding globulin level. Mincey's original ETR procedure (*4*) was encumbered by a laborious and imprecise extraction of the thyroxine from serum with alcohol, followed by the addition of a small amount of the patient's serum to the final reaction mixture to account for variations in levels of binding proteins. Instead of reporting an absolute value for the serum T_4(T_4D) (*5*), and T_3 uptake (T_3U), the results of the test were expressed as a ratio of the radioactive counts in the patient tubes to the counts in a tube containing a "euthyroid reference serum." This value was designated the effective thyroxine ratio.

Mincey later modified the original procedures by using a dilute solution of hydrochloric acid to free the thyroxine from binding proteins (*6*). The direct use of the HCl-denatured serum in the competitive protein binding assay eliminated the need to extract and centrifuge a precipitate or add patient's serum to the reaction mixture, while improving the speed and simplicity of the assay. Several manufacturers of competitive protein binding radioassay kits for T_4 have modified their products so that the results of the assay may be expressed as a ratio to a reference serum, or as a normalized or compensated T_4 (*7–9*). Most competitive protein binding kits for T_4 can be adapted to use either the alcohol extraction plus addition of untreated serum, or the acid-treated serum method for measurement of either ETR or normalized T_4. Since the reported parameter is a ratio of the results obtained with the patient and euthyroid reference serum, the ETR is therefore very dependent on the nature of the selected reference serum.

The statistical relationship between ETR and other thyroid function tests such as T_4D, FTI, and free T_4 has been reported by several investigators (*10–13*). However, the dependence of ETR on the individual parameters of T_4D and T_3U in the reference serum has not been described. This lack of description has limited the ETR's acceptance.

Materials and Methods

Thyroxine concentrations (T_4D) in serum were determined by a competitive protein binding radioassay using the Thyrox-I-Take kit (Dade, Division American Hospital Supply Corp., Miami, FL). Resin T_3 Uptake (T_3U) values were determined using Triosorb-M 125 (Abbott Diagnostics, North Chicago, IL). These materials were

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used according to the manufacturer's instructions. Control sera (Monitrol I and II, unassayed) were obtained from Dade. Hydrochloric acid solutions were prepared from distilled water and reagent-grade concentrated acid. The Thyrox-I-Take kit was used to determine the effective thyroxine ratios with acid-denaturation of the serum, following the protocol given.

One hundred and twenty-five μl of patient, euthyroid reference, or control serum was mixed with 125 μl of 0.5 N HCl and allowed to stand for 20 min. Then, 175 μl of this mixture was aspirated with the sampling probe of an automatic pipettor/dilutor (Micromedic Systems, Horsham, PA) and expelled into a polypropylene tube with 5 ml of the I-125-labeled thyroxine/TBG/buffer reagent supplied with the Thyrox-I-Take kit. After incubation for 30 min, 80 mg of ion exchange resin, supplied in preweighed vials, was added sequentially to the reaction tubes at timed intervals. The tubes were capped, transferred to a rotator, and arranged to produce a tumbling of the solution and resin beads. After 15 min, the tubes were removed, and the solution was poured from the resin in the same sequence and time interval used to add the resin. A plunger device supplied with the kit was used to insure that no resin was decanted. The resin beads were washed with 5 ml of distilled water and the wash decanted. The resin beads were counted for 1 min, which assured an accumulation of at least 2×10^4 counts. All counting was done in a Searle 1185 gamma counter equipped with a preset I-125 window and a 3-in. crystal. We calculated the ETR as cpm patient/cpm reference sera. This inversion of Mincey's calculation is necessary since he counted TBG-bound label and we counted unbound label. This maintains the logical relationship of high ETR in hyperthyroidism and low ETR in hypothyroidism.

Sera from patients on whom T_4D and T_3U tests had been requested by an attending physician were also subjected to the acid denaturation ETR procedure. The data we present were retrospectively selected from among these samples.

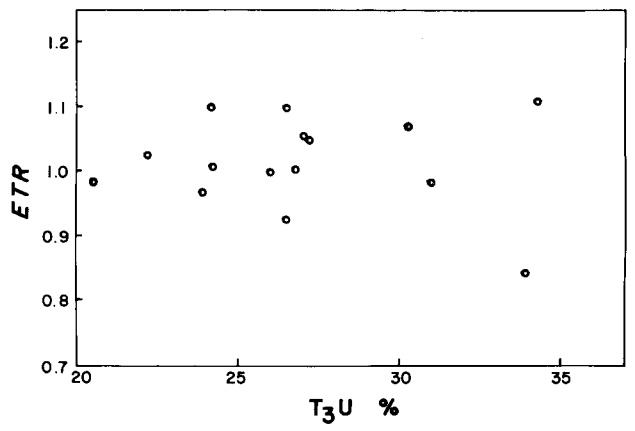

FIG. 1. Sensitivity of ETR of an euthyroid serum (Monitrol I, T_4D = 10.7; T_3U = 31) to the value of T_3U of reference sera. Ordinate equals observed ETR of Monitrol relative to reference sera. Abscissa equals T_3U of reference sera (note all have T_4D equal to 9.0 ± 0.5); n = 15.

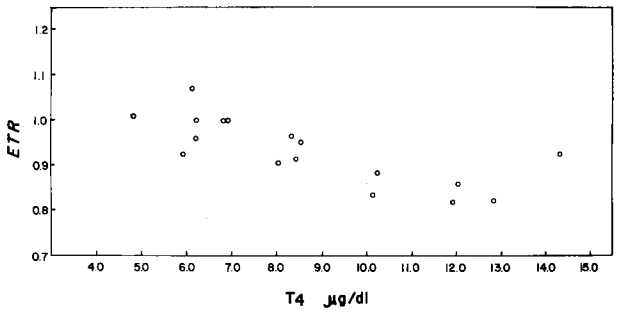

FIG. 2. Sensitivity of ETR of euthyroid serum (Monitrol I, T_4D = 10.7, T_3U = 31) to the value of T_4D of reference sera. Ordinate equals observed ETR of Monitrol relative to reference sera. Abscissa equals T_4D of reference sera (note all have T_3U equal to 30 ± 1); n = 17.

Results and Discussion

Since the ETR is a ratio of a patient result to a euthyroid reference serum result, the nature of this reference serum will have a profound effect on the individual patient values as well as the range of values observed in a normal population. The data presented in Figs. 1 and 2 will aid the laboratorian in selection of an acceptable reference serum. To facilitate interpretation of the individual parameters, T_4D, T_3U, and ETR, precision data are presented in Table 1.

The ETR of Monitrol I (which has $T_4D = 10.7$; $T_3U = 31$; $FTI = T_4D \times T_3U = 3.32$; normal ranges $T_4D = 5$–13 μg/dl; T_3U = 24–35%; and FTI = 1.2–4.5) was calculated against multiple reference sera and is illustrated in Fig. 1. All reference sera had the same $T_4D = 9.0 \pm 0.5$ but different T_3U. The ordinate is the ETR value of Monitrol I and the abscissa is the T_3U of the reference serum used to calculate this ETR. There is no systematic variation in the ETR of the Monitrol despite a sizeable change in the relative thyroxine binding protein level of the reference sera used in the calculations.

Careful examination of data in Fig. 1 indicates the free thyroxine index ($T_4 \times T_3U/100 = FTI$) of the various samples ranges from $9 \times 21/100 = 1.89$ to $9 \times 34/100 = 3.06$ (normal: 1.20–4.55). Since all the FTI values are normal, it is encouraging that all but one of the observed

TABLE 1. Precision

Parameter	Mean	sd	cv
T_3U (%)	27.8	1.5	5.4
T_4D (μg/dl)	9.6	0.66	6.9
ETR	1.01	0.02	2.0

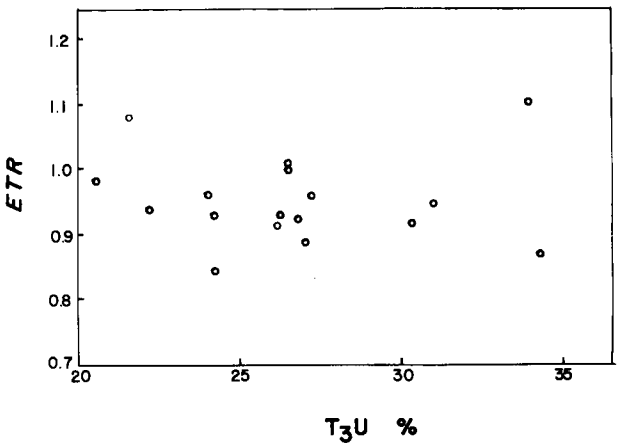

FIG. 3. Effect of T3U on observed ETR. Ordinate equals observed ETR relative to euthyroid Monitrol. Abscissa equals T3U of the sera (note all have T4D equal to 9.0 ± 0.5); n = 17.

ETR's are normal (0.89–1.09)*(4)*. These data indicate that the ETR is not systematically related to the thyroxine binding globulin concentration of the reference serum. Since T_3U and ETR methods have been designed so that binding of T_4 and T_3 by thyroxine binding prealbumin (TBPA) is inhibited *(1)*, alterations in TBPA will not affect these parameters.

The graph in Fig. 2 shows the ETR of Monitrol I when calculated with reference sera having a constant T_3U of 30% ± 1 and variable T_4D. The ordinate is again the ETR value of Monitrol I; the abscissa is the T_4D of the reference serum used to calculate this ETR. As demonstrated in Fig. 2, the ETR is systematically and significantly dependent on the T_4D of the reference sera. Therefore, the clinical reference or normal range will be strongly dependent on the T_4D of the reference sera, and careful selection of reference sera is necessary to ensure comparability of ETR values measured using different reference sera. If a given laboratory was using a reference serum with T_4D = 8.0, T_3U = 30, and FTI = 2.40, and changed to one with T_4D = 10.0, T_3U = 30, and FTI = 3.0, a shift would occur in the reported ETR values. Conversely, as indicated in Fig. 1, the T_3U of the reference serum is of lesser importance. To ensure proper standardization over time, the laboratory must overlap pools of reference sera, selecting one with a T_4D, T_3U, and ETR near the population mean.

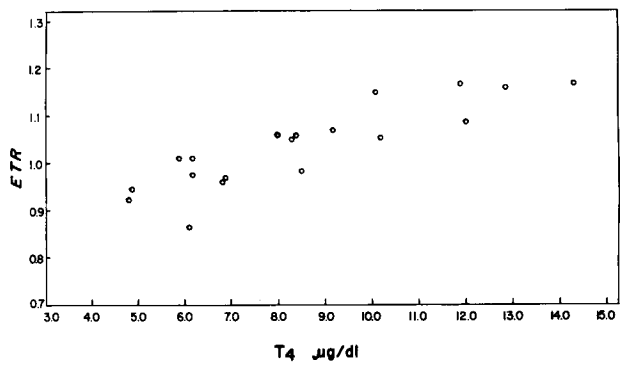

FIG. 4. Effect of T4D on observed ETR. Ordinate equals observed ETR relative to euthyroid Monitrol. Abscissa equals T4D of sera (note all T3U equals 30 ± 1); n = 19.

The clinical utility of the ETR has been questioned since neither the actual T_4D or T_3U are known. The ETR and free thyroxine (FT_4) and free thyroxine index (FTI) are highly correlated as previously noted *(10–13)*. Therefore, we have not repeated this work, but have tried to clarify the relationship of ETR to the individual parameters of T_4D and T_3U.

It has been previously shown that the free sites available on thyroxine binding globulin are inversely related to the T_3U, but that this correlation is poor in euthyroid sera *(14)*. To demonstrate the relationship of ETR to T_3U, a group of sera were selected with the same T_4D (9.0 ± 0.5) and variable T_3U. Within this group, as the T_3U increases the number of free sites on TBG declines; therefore, one predicts that the sera will have increasing FT_4 and ETR as the T_3U rises. However, Fig. 3 demonstrates that this is not observed, and ETR does not systematically change with changing T_3U. The insensitivity of ETR to T_3U can be explained because free TBG sites and T_3U are poorly correlated in euthyroid sera *(14)*, and all these sera are euthyroid; the relative imprecision of the T_3U diminishes the correlation; and physiological equilibrium mechanisms would be expected to maintain the free T_4 concentration relatively constant despite small variations in the thyroxine binding globulin level.

To study the sensitivity of ETR to T_4D, a group of sera with the same T_3U (30 ± 1) and variable T_4D were analyzed. All of these sera have nearly identical numbers of free sites on thyroxine binding globulin, since both the T_3U and ETR are insensitive to TBPA *(1)*. Therefore as the T_4D increases in this group of sera, the relative saturation of TBG, the free T_4, and the ETR would be expected to increase. Figure 4 demonstrates this effect. As the T_4D increases, there is a systematic rise in ETR.

Since the use of traditional parameters such as total T_4 and T_3U are well established, the clinical efficacy of the ETR needs to be validated. Previous reports have demonstrated that ETR is an excellent predictor of the free thyroxine or the free thyroxine index. The data presented here document the care required for adequate standardization of the ETR over extended time periods, and the relationship of the ETR to the individual parameters, T_4D, and T_3U.

Acknowledgments

This paper was presented in part at the 29th Annual Meeting of the American Association for Clinical Chemistry, Chicago, June 17–22, 1977.

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