jnm/ CONCISE COMMUNICATION

AN IN VITRO THYROID FUNCTION TEST WITHOUT ALCOHOL EXTRACTION

E. K. Mincey, M. E. Wilcox, and R. T. Morrison

University of British Columbia, Vancouver General Hospital, Vancouver, B.C., Canada

A simplified method for performing a dual-function competitive binding test for assessing thyroid function without the need for ethanol extraction and separate addition of unextracted serum aliquots has been developed.

Competitive protein-binding tests for serum thyroxine using thyroxine-binding globulin (TBG) as the binder require some treatment of patient serum samples to remove the endogenous thyroxine (T_4) and to denature or remove the native TBG. The most widely used reagent for this purpose is ethanol which precipitates the serum proteins and causes release of T_4 into the supernatant liquid. Most T_4 procedures require drying of an aliquot of the alcoholic extract before testing whereas at least two commercial methods allow addition of a portion of the alcoholic extract directly into the reaction vial (*1,2*). Mincey, et al (*3–5*) described a new test concept that embodied the principles of the T_4 test and the triiodothyronine (T_3) resin uptake test in one procedure. This concept made it possible to ultilize one simple test for evaluation of thyroid function which could compensate for the alterations of concentration of TBG found in pregnant women and in women taking contraceptive medication. This test has shown excellent correlation with absolute free thyroxine levels and has been shown to have a high degree of correlation with the clinical diagnosis when used as a single screen of thyroid function (*6,7*). As in the T_4 test, however, patient serum must be extracted with ethanol to obtain the T_4 component while an additional small portion of unextracted patient serum serves as the unsaturated TBG component. This requires two separate additions and necessitates measuring 5-μl quantities of serum. We have devised a new and simpler method of performing the dual-function test without alcohol extraction and without separate addition of unextracted serum aliquots.

METHOD

Radioactive reagent for the dual-function test was obtained from Mallinckrodt/Nuclear, St. Louis, Mo. [effective thyroxine ratio (ETR)]. Reagents intended for T_4 assay may also be used (Resomat T_4, Mallinckrodt/Nuclear). Each vial contains 4.0 ml of 0.1 *M* barbital buffer, pH 8.6 containing about 2.5 μCi of ^{125}I T_4 and 0.5 ml of processed normal serum per 100 ml of solution. Alternatively, any radioactive reagent solution approximating the above may be used after some preliminary experiments by the user. We obtained the commercially prepared reagent for the sake of expediency.

An aliquot of test serum from each patient (0.2 ml) and an aliquot of reference serum from the ETR kit (0.2 ml) was added to a series of labeled polystyrene tubes (12 × 75 mm, Falcon plastics) and 0.2 ml of 0.5 *N* HCl was added to each tube also. After vortexing, the tubes were allowed to stand for 10 min and then 0.2 ml from each tube was transferred to a vial containing radioactive reagent. A resin strip furnished with the kit was added to each vial and incubation was carried out on a rotator (12–14 rpm) at 23°C for 60 min. At the end of this time, the resin strips were removed and discarded and the vials counted in a well scintillation counter. The test results were expressed as a ratio of the cpm reference serum vial to the cpm test serum vials.

As an alternative procedure, 0.1 ml of serum and 0.1 ml of 0.5 *N* HCl may be added to polystyrene tubes and then 4.0 ml of radioactive reagent added directly to the tubes. The other steps in the procedure are unchanged.

Received April 16, 1974; revision accepted June 18, 1974.

For reprints contact: E. K. Mincey, Div. of Nuclear Medicine, Vancouver General Hospital, Vancouver 9, B.C., Canada.

TABLE 1. EFFECT OF SERUM TREATMENT ON REMOVAL OF ^{125}I T_4 FROM RADIOACTIVE REAGENT SYSTEM

Patient	Serum + 0.1 N HCl* (%)	Serum + 0.5 N HCl* (%)	Serum + 1.0 N HCl* (%)	0.1 ml serum only (%)
Normal	64†	63	65	86
Pregnant	61	62	66	90
Pregnant	63	66	68	90
Hypothyroid	80	75	77	89
Hyperthyroid	54	53	57	84

* Serum and acid (1:1) incubated together for 10 min before adding to radioactive reagent system.

† All values expressed as percentage of initial counts remaining in vial after rotation. Each number represents the mean of triplicate determinations.

In two other experiments, 0.1 *N* and 1.0 *N* acetic acid was substituted for hydrochloric acid without any other alteration in the method.

Duplicate estimates of ETR were determined in serum from 60 euthyroid adults, 10 pregnant women, 20 hypothyroid patients, and 10 hyperthyroid patients and were compared with the test results obtained by the method described herein.

RESULTS AND DISCUSSION

The results obtained with the ETR or other dual-function in vitro thyroid function tests are explicable on the basis that when only extracted T_4 is added to the system, the results vary solely with the concentration of T_4 present in the test serum. Similarly, test values obtained with the addition of only unextracted serum vary inversely with the relative degree of saturation of TBG. It follows, then, that the ETR test results obtained with the addition of both extracted T_4 and an appropriate aliquot of the same patient's serum are dependent on both the concentration of serum T_4 and the degree of saturation of TBG. Consequently, the concentration of T_4 available for competition in the test system itself will be approximately the same for all euthyroid patients irrespective of binding protein abnormalities and will be appropriately increased or decreased in hyperthyroid and hypothyroid patients. The data in Table 1 show that when only serum is added to the radioactive reagent system, the radioactive T_4 remaining in the vial after exposure to the anion resin strip is related solely to the degree of saturation of TBG of the individual serum samples. After exposure to hydrochloric acid, however, the same amount of serum (0.1 ml) exerts a much more profound effect on the test system so that the amount of ^{125}I T_4 ultimately displaced is proportional to both the T_4 concentration of the serum and the degree of unsaturation of TBG. This is evident from the euthyroid pregnancy sera data since both samples had a serum thyroxine value above the normal range but the effect on the present test system is the same as that produced by euthyroid, normal serum. It appears that lowered pH causes the release of T_4 from the native TBG binder and also partially denatures the TBG in serum so that the overall effect is exactly the same as when the alcohol-extracted T_4 and the patient TBG are added as separate components. That is to say, the ratio between total thyroxine and TBG concentration in the individual patient serum is maintained when the serum is exposed to acid. It should be apparent that if some standard serum samples with known thyroxine values are assayed along with the test sera, it is possible to construct a curve and report out corrected T_4 values for the unknowns.

We have performed other experiments in which the serum–acid mixture was allowed to stand for various periods up to 120 min before removal of an aliquot for testing. The results in Table 2 show that more radioactivity is removed with increasing exposure time of the serum to the acid. This may indicate more complete removal of endogenous T_4 or progressive denaturation of TBG with standing at lowered pH. If the reference and test serum are incubated for the same time, however, the final result, expressed as a ratio, is essentially the same for all time periods tested. In addition, a plateau is reached after 10 min so that elapsed time in transferring aliquots to the reaction vials will not become a factor.

Table 3 illustrates some representative comparative values on 25 serum samples chosen from the first 100 samples tested. There is no doubt that both methods produce virtually identical results on the same serum samples. The acid method obviates the need for centrifugation and for pipetting separate aliquots of alcoholic extract and whole serum and

TABLE 2. RELATIONSHIP BETWEEN RADIOACTIVITY REMAINING IN REAGENT VIAL AND INCUBATION PERIOD OF SERUM–ACID MIXTURE

Incubation time (min)	cpm reference serum	cpm patient serum	cpm reference/ cpm patient ratio
0	18,901	18,273	1.03
5	14,805	15,355	0.96
10	14,366	14,411	1.00
20	14,330	14,056	1.02
30	13,941	14,128	0.99
60	13,690	13,813	0.99
120	13,411	13,208	1.01

TABLE 3. COMPARISON OF ETR VALUES AND RESULTS OF THE ACID EXTRACTION METHOD OF SOME REPRESENTATIVE SERUM SAMPLES

Patient No.	Diagnosis	ETR value*	Acid extraction method
1	Euthyroid	1.00	0.99
2	Euthyroid	1.00	1.03
3	Euthyroid	1.01	1.02
4	Euthyroid	1.00	0.99
5	Euthyroid	0.98	0.96
6	Euthyroid	1.01	0.97
7	Euthyroid	1.00	0.98
8	Euthyroid	1.10	1.09
9	Euthyroid	1.01	1.01
10	Euthyroid	0.95	0.98
11	Pregnant	0.99	0.99
12	Pregnant	0.97	0.96
13	Pregnant	0.96	0.93
14	Pregnant	0.97	0.98
15	Pregnant	0.96	0.96
16	Hypothyroid	0.83	0.78
17	Hypothyroid	0.87	0.80
18	Hypothyroid	0.83	0.81
19	Hypothyroid	0.80	0.84
20	Hypothyroid	0.85	0.87
21	Hyperthyroid	1.53	1.43
22	Hyperthyroid	1.21	1.19
23	Hyperthyroid	1.24	1.23
24	Hyperthyroid	1.14	1.13
25	Hyperthyroid	1.36	1.34

* Normal range is 0.89–1.10.

offers a very reproducible method of performing a dual-function test such as the ETR. The acid effect is apparently attributable to lower pH since the test results in Table 1 are identical using from 0.1 to 1.0 *N* HCl. We have also determined that acetic acid of 0.1 *N* produces no effect but 1.0 *N* HAC produces results identical to the HCl values on the same serum samples.

Finally, treating serum with acid overcomes some of the disadvantages of working with alcoholic extracts, especially if the method calls for adding some of the extract directly to the radioactive reagent system. As is well appreciated, ethanol has a deleterious effect on the TBG binder in the test reagent. With the commercial reagents used in this study, rotation of the vials for 1 hr with the resin strip alone resulted in removal of 30% of the ^{125}I T_4. Rotation with 0.3 ml of 66% ethanol plus the resin strip removed 50% of the radioactivity. Thus, only 50% of the ^{125}I T_4 initially present in the test system is directly responsive to T_4 present in the alcoholic extract. In contrast, rotation of the vials with 0.1 ml of 0.5 *N* HCl and 0.1 ml of H_2O plus the resin strip removed only 30% of the radioactivity, which is attributable to the resin effect alone.

REFERENCES

1. THORSON SC, TSUJIKAWA R, BROWN JL, et al: Evaluation of a simplified method for determining serum thyroxine by competitive protein binding analysis. *Acta Endocrinal* 64: 630–636, 1970

2. JOSEPH K, MAHLSTEDT J: The usefulness of the Thyopac-4 test as a screening method and the first comparisons between the FTI and ETR principle. *Nucl Compact* 3: 78–80, 1972

3. MINCEY EK, THORSON SC, BROWN JL, et al: A new parameter of thyroid function—the effective thyroxine ratio. *Clin Res* XIX, 774, 1971

4. MINCEY EK, THORSON SC, BROWN JL: A new in-vitro blood test for determining thyroid status—the effective thyroxine ratio. *Clin Biochem* 4: 216–221, 1971

5. MINCEY EK, THORSON SC, BROWN JL, et al: A new parameter of thyroid function—the effective thyroxine ratio. *J Nucl Med* 13: 165–168, 1972

6. THORSON SC, MINCEY EK, MCINTOSH HW, et al: Evaluation of a new in-vitro blood test for determining thyroid status: the effective thyroxine ratio. *Br Med J* 2: 67, 1972

7. WELBY ML, O'HALLORAN MW, MARSHALL J: A comparison of effective thyroxine ratio and free thyroxine in serum. *Clin Chim Acta* 45: 255–259, 1973