United States Patent [19]

Ashkar

[11] **3,850,577**

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[54] **RAPID EXTRACTION METHOD FOR SERUM THYROXINE**

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[52] **U.S. Cl.** ..... **23/230 B**

[51] **Int. Cl.** **G01n 33/16**

[58] **Field of Search** ..... 23/230 B; 424/1

[56] **References Cited**

UNITED STATES PATENTS

3,702,821	11/1972	Fernandez	23/230 B
3,745,211	7/1973	Brown et al.	23/230 B
3,775,615	11/1973	Eisentraut	23/230 B
3,799,740	3/1974	Mincey	23/230 B

Primary Examiner—R. E. Serwin

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[57] **ABSTRACT**

A method for determining total thyroxine by the competitive protein binding analysis procedure and in which an acid buffer is employed to liberate the bond thyroxine from the serum proteins to make the thyroxine available for competitive binding. The buffer greatly reduces extraction time in comparison to the use of alcohol, conventionally used in such procedures, and comprises a glutamate - salicylate mixture.

5 Claims, 4 Drawing Figures

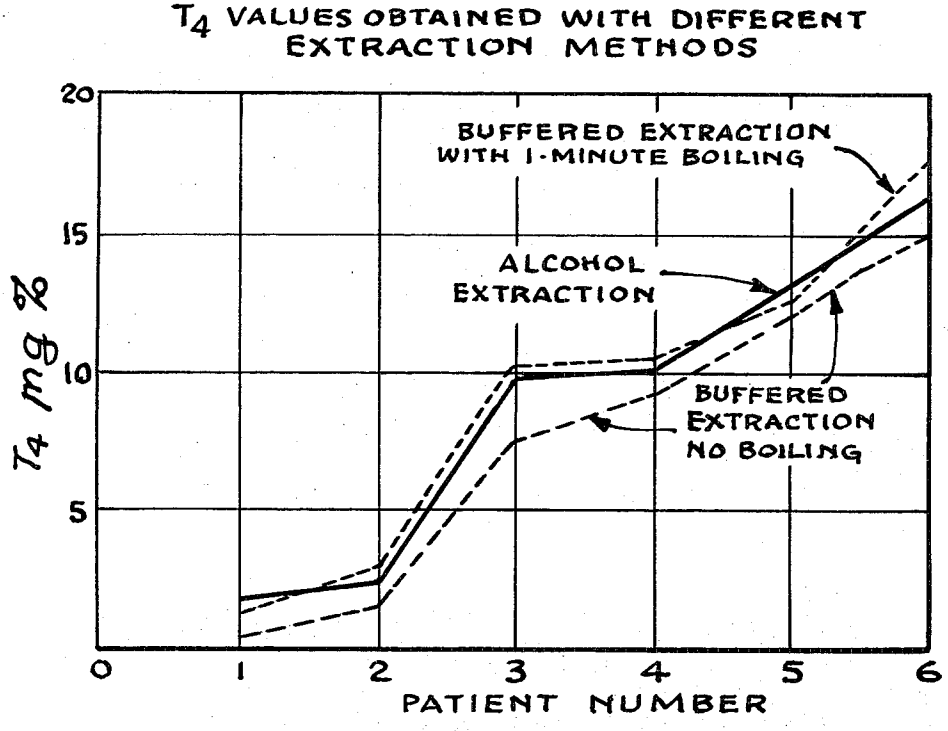
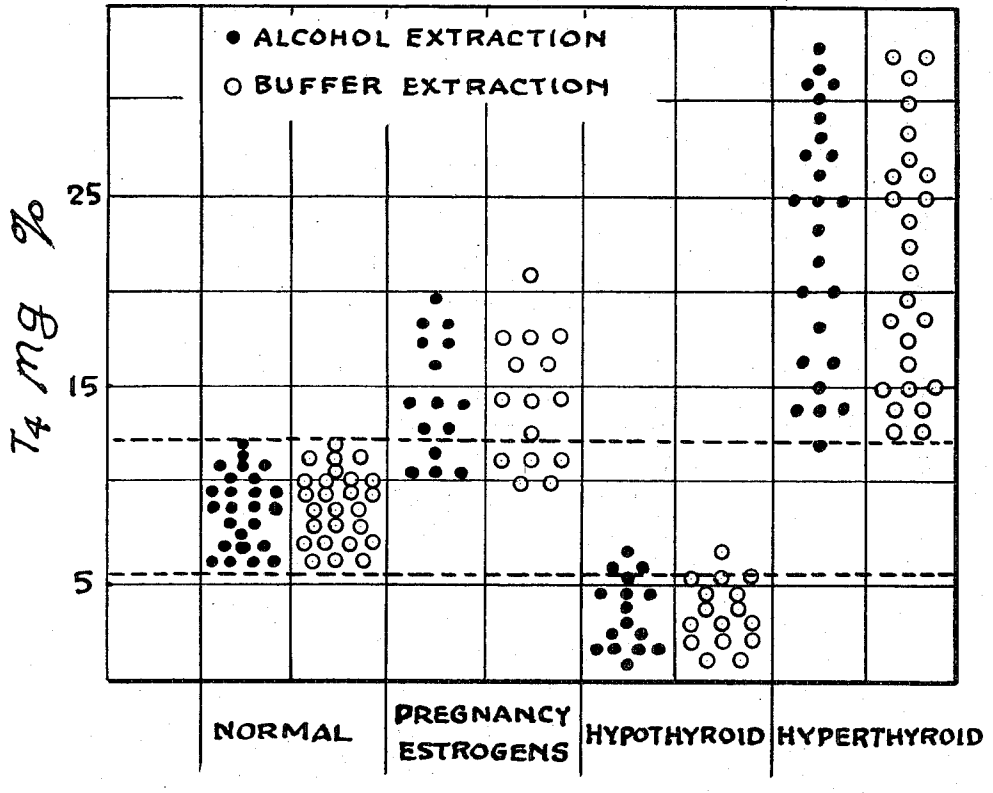

T_4 VALUES OBTAINED WITH DIFFERENT EXTRACTION METHODS
BUFFERED EXTRACTION WITH 1-MINUTE BOILING
ALCOHOL EXTRACTION
BUFFERED EXTRACTION NO BOILING
T4 mg %
20
15
10
5
0
1
2
3
4
5
6
PATIENT NUMBER

FIG. 1

FIG. 2


• ALCOHOL EXTRACTION
○ BUFFER EXTRACTION
T4 mg %
25
15
5
NORMAL
PREGNANCY ESTROGENS
HYPOTHYROID
HYPERTHYROID

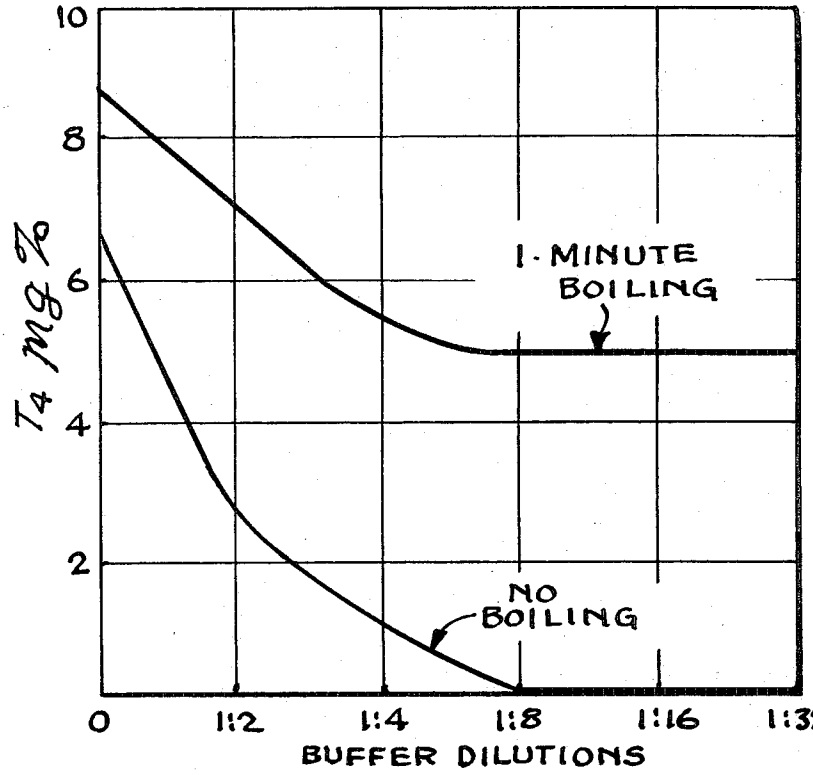


FIG.3

THE EFFECT OF BUFFER DILUTIONS ON THYROXINE EXTRACTION FROM NORMAL SERUM

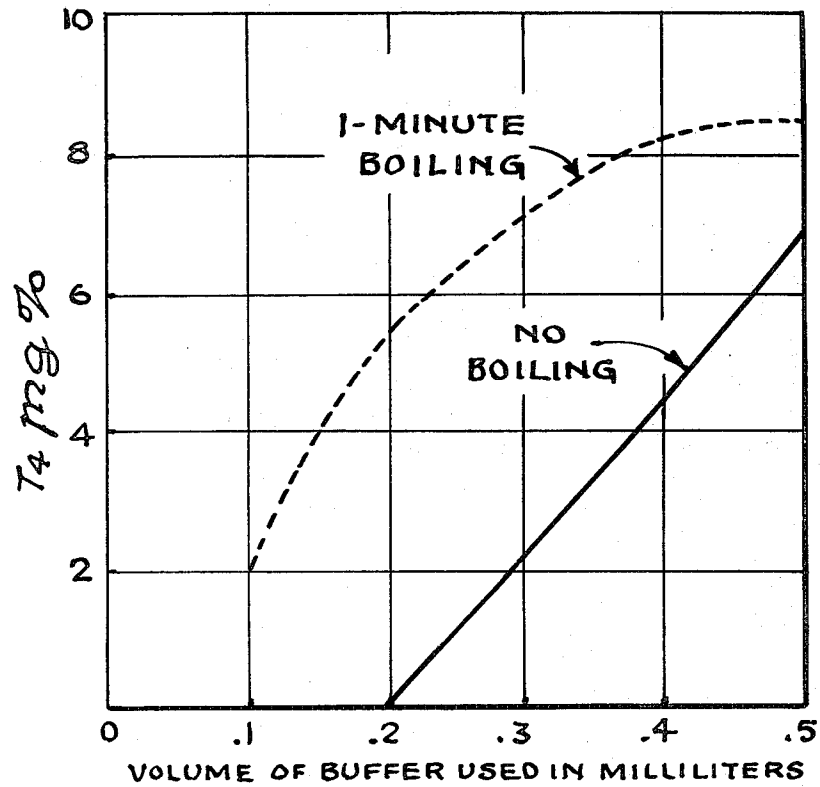


FIG.4

THE EFFECT OF INCREASING THE AMOUNT OF BUFFER ON THYROXINE EXTRACTION FROM NORMAL SERUM

RAPID EXTRACTION METHOD FOR SERUM THYROXINE

BACKGROUND OF THE INVENTION

The primary function of the thyroid gland is the regulation of cellular metabolism through the synthesis and secretion of two hormones, triiodothyronine (T3) and tetraiodothyronine (thyroxine or T4). These hormones are primarily bound by three plasma proteins once they are secreted from the thyroid. These are thyroxine binding globulin (TBG) to which binding is the strongest; prealbumin to which binding is of intermediate affinity; and albumin to which binding is relatively weak. One of the first in vitro tests which was developed to measure thyroid activity, specifically total serum thyroxine, using the principal of competitive protein binding is described in Clin. Chem. Acta 5:453, 1960 by R. P. Ekins. Ekins extracted the thyroxine from serum by precipitation with butanol. Murphy in Nature, 201:679, 1964 and Murphy and Patee, in Journal Clin. Endocrinology and Metabolism, 24:187, 1964 described a more rapid and simple method of total serum thyroxine measurement based on the same principal, which they refer to as "competitive protein binding analysis." In this procedure, a single ethanol extraction is used to separate the thyroxine from the serum. Loose anion exchange resin was employed to separate TBG bound thyroxine from unbound thyroxine. An important requirement in such tests is a simple, quick and reproducible method of separating TBG bound thyroxine from unbound thyroxine after equilibrium has been obtained in test conditions. U.S. Pat. Nos. 3,206,602 and 3,376,114 describe a test method and apparatus employing a resin sponge as a means of separating bound from unbound thyroxine. Such resin sponges have finely divided ion exchange resin dispersed within the sponge. Unbound thyroxine is quickly and quantitatively bound to the resin sponge while TBG bound thyroxine is not.

The "competitive protein binding analysis" method for the determination of total serum thyroxine is based upon the in vitro competition between thyroxine and tracer ^{125}I thyroxine for the binding sites of thyroxine binding globulin. The ion exchange resin acts as a secondary binding site for the unbound thyroxine. The ion exchange method measures the amount of thyroxine which becomes irreversibely bound to the resin-sponge. This amount depends on the disassociation constant of thyroxine binding with serum proteins, the number of binding sites on the protein, the pH, temperature and incubation time in the test conditions. The competitive protein binding analysis technique requires the use of ethanol to liberate bound thyroxine from serum proteins to make it available for competitive binding. The extraction time for liberating bound thyroxine using ethanol ranges between 15 to 45 minutes and requires anywhere between 3 to 4 steps to achieve an 85 percent extraction efficiency. A simpler and more rapid thyroxine extraction method is therefore desirable since measurement of total thyroxine in serum has become one of the primary in vitro tests for evaluating thyroid function and has gained great acceptance.

SUMMARY OF THE INVENTION

It has been found that an acid buffer comprising a glutamate - salicylate mixture is an excellent agent for rapid thyroxine extraction. Preferably, the buffer comprises a mixture of glutamic acid and sodium salicylate having a pH of 3.5. When 0.1 ml. of serum is mixed with 0.5 ml. of the buffer, 85 percent of the thyroxine in the serum will be instantaneously extracted. This is comparable to the extraction efficiency of the conventional alcohol extraction procedure. If the serum-buffer mixture is heated, an extraction efficiency approaching 100 percent results. It is believed that the acid buffer functions by preventing protein coagulation. Substitution of an acid buffer for the alcohol in the extraction step of the competitive protein binding analysis method has the advantages of speed, simplicity, low cost, fewer steps, greater extraction efficiency and excellent reproducibility.

DRAWINGS

The method of the present invention will be better understood with reference to the following description and drawings in which:

FIG. 1 is a graph illustrating serum thyroxine values (T4) obtained with different extraction methods, i.e., alcohol or buffer.

FIG. 2 is a graph comparing serum thyroxine values obtained with alcohol extraction and buffer extraction methods in normal, hypothyroid and hyperthyroid patients.

FIG. 3 is a graph illustrating the effect of buffer dilution on thyroxine extraction from normal serum.

FIG. 4 is a graph illustrating the effect on thyroxine extraction of increasing the amount of buffer with respect to the amount of serum sample.

DETAILED DESCRIPTION

The substitution of an acid buffer for the alcohol as an extraction agent to liberate bound thyroxine from serum proteins in the competitive protein binding analysis method for determining total serum thyroxine has been found to produce essentially instanteous extraction. A buffer comprising glutamic acid and sodium salicylate, 12 grams of glutamic acid and 0.04 grams of sodium salicylate per liter, is preferred. The extraction ability of 0.5 ml. of this buffer was tested using 0.1 ml. of serum and compared to the use of alcohol. The effect of heating as well as increasing the amount of buffer, and buffer dilution were also evaluated. The reproducibility of the buffer in the hypo and hyperthyroid hormone levels was also determined. Thyroxine levels were obtained by mixing the serum and buffer mixture with thyroxine binding globulin fully saturated with labeled thyroxine. After competitive binding had taken place, the bound and free labeled thyroxine were separated by an anionic resin and the percent resin bound I^{125} thyroxine was calculated. The final results were obtained by comparing the samples radiometrically to thyroxine standards.

EXAMPLE 1

Using the apparatus and method disclosed in U.S. Pat. Nos. 3,206,602 and 3,376,114, 0.5 ml. of buffer was added to 0.1 ml. of serum and the mixture placed in an ice bath for 5 minutes. One milliliter of I^{125} labeled thyroxine binding globulin reagent was then added together with an ion exchange resin-sponge and the mixture incubated in ice water for 1 hour. Using a scintillation counter, the radioactivity of the combined mixture is determined and, after incubation, the fluid

is aspirated from the tube and the sponge is washed with water. The radioactivity remaining on the sponge is determined and the percent sponge uptake for each sample is obtained by dividing the final count by the initial count.

The spontaneous extraction efficiency of the acid buffer was found to be 85 percent which is comparable to the extraction efficiency of alcohol. When the serumbuffer mixture was boiled, the extraction efficiency approached 100 percent (See FIG. 1). Increasing amount of acid buffer in comparison to the serum sample improved the extraction ability progressively both with and without boiling. Spontaneous extraction did not occur until the volume of buffer exceeded 0,2 ml. and reached the 85 percent extraction efficiency with 0.5 ml. of buffer. Thus optimum extraction was found to occur when 0.1 ml. of serum was mixed with 0.5 ml. of buffer and the mixture boiled for one minute (See FIG. 4). The extraction efficiency of the acid buffer was found to decrease when the buffer was diluted. With heating the extraction efficiency was found to drop to zero when the dilution reached 1:8, while with the heating technique, the efficiency dropped to a plateau after a 1:8 dilution. The extraction efficiency of the acid buffer was found to be consistent in low, normal and elevated thyroxine levels as evidenced by the thyroxine obtained in the case of two hypothyroid, two normal and two hyperthyroid patients as compared to the use of alcohol for the extraction of thyroxine.

As is apparent from FIG. 4, using 0.1 ml. of serum, the ratio of buffer to serum is preferably maintained from between 1:1 to 5:1. Likewise, (see FIG. 3) the buffer is preferably used at a concentration of 12 grams per liter of glutamic acid and 0.04 grams per liter of sodium salicylate but is effective down to a dilution of 1:8, the concentration of ingredients ranging from 1:33 to 12 grams per liter of glutamic acid and 0.004 to 0.04 grams per liter of sodium salicylate.

What is claimed is:

1. In a method of determining the total serum thyroxine in a serum sample, said method comprising:
extracting the thyroxine from the thyroxine binding globulin in the serum sample;
mixing a tracer amount of thyroxine globulin saturated with radioactively labeled thyroxine with the thyroxine extracted from the thyroxine binding globulin in the serum sample;
placing an ion exchange resin in intimate contact with said mixture;
incubating the combined mixture and ion exchange resin;
measuring with suitable detecting means the initial radioactivity of the combined mixture and ion exchange resin;
removing the mixture; and
measuring with suitable detecting means the residual radioactivity in the ion exchange resin;
the improvement comprising extracting the thyroxine from the thyroxine binding globulin in a serum sample by placing a buffer in contact with said serum sample, said buffer comprising a glutamate salicylate mixture.

2. The method of claim 1 wherein said buffer comprises a mixture of glutamic acid and sodium salicylate, the concentration of glutamic acid ranging from about 1:33 to about 12 grams per liter and the concentration of sodium salicylate ranging from about 0.004 to about 0.04 grams per liter.

3. The method of claim 2 wherein said buffer comprises a mixture of 12 grams per liter of glutamic acid and 0.04 grams per liter of sodium salicylate.

4. The method of claim 3 wherein the ratio of buffer to serum sample is from 1:1 to 5:1.

5. The method of claim 1 wherein the serum-buffer mixture is heated prior to adding the radioactively labeled thyroxine binding globulin.

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