METHOD OF MEASURING SERUM THYROIDINE

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Field of Search............... 250/83 R, 83 SA, 106 T; 23/230 B

ABSTRACT

A new and improved in vitro concept in measuring serum thyroxine (T-4) is disclosed which employs an alkaline cross-linked dextran gel (Sephadex) column to dissociate and separate the T-4 from the serum protein in a single operation. An isotope dilution technique combined with saturation analysis is used to estimate the T-4 content in serum.

7 Claims, No Drawings
METHOD OF MEASURING SERUM THYROIDINE

BACKGROUND OF THE INVENTION

The thyroid gland is extremely important in the animal body because of its effect upon the basal metabolic rate. This effect is regulated by the thyroid hormone which is released in response to nervous or hormonal stimuli. Thyroxine enters the circulatory system and acts directly upon the cell or indirectly upon other hormonal systems. Abnormal activity of the thyroid is a common malady in humans. In hypothyroidism, the body has decreased thyroid activity which is manifest by such diseases as cretinism and myxedema. Hyperthyroidism is a state of excessive thyroid activity in which one becomes nervous, develops an increased pulse rate and sometimes goiter.

Thyroid deficiency was associated with a reduced metabolic rate as early as 1895 and several systems based on basal metabolic rate were devised for estimating thyroid activity. However, such systems were not reliable, so more direct and precise methods were sought. In 1896, iodine was discovered in thyroid extract but the relationship between blood iodine level and thyroid function was not established until 1933. This led to the use of protein-bound iodine as a means of estimating thyroid function, and by 1955 the PBI test was standard for checking thyroid activity. It soon became apparent that this test was influenced by the administration of other iodine containing compounds to the patient. Thus, the butanol-extractable iodine (BEI) procedure was devised which gave better correlation between serum iodine levels and the clinical findings but was likewise non-specific for T-4.

A major advance in T-4 analysis occurred in 1959 when Galton et al., Biochem. J., 72, 310 (1959) liberated T-4 from serum protein by hydrolysis and separated it from other iodine compounds by passage through a resin column. Later, Pileggi et al., J. Clin. Endocrinol. Met. 21, 1272 (1961) developed this chromatographic procedure into a clinically usable method.

In 1964, Murphy et al., J. Clin. Endocrinol. Met. 24, 187 (1964) developed a T-4 assay based upon competitive protein binding and isotopic dilution which required the initial extraction of T-4 from the serum with alcohol followed by centrifugation and drying. Although this test was highly specific, only 80 percent of the T-4 could be recovered from the serum.

More recently, U.S. Pat. No. 3,471,533 set forth still another column chromatography T-4 assay in which an auxion-exchange resin is adjusted to an alkaline pH of about 12 to disassociate the thyroxine from its albumin and globulin carriers. The diluted serum solution is then poured onto the resin wherein proteins, amino acids, thyroxine, iodotyrosine and iorganic iodine are adsorbed. Successful washes with acetic acid buffer isopropyl alcohol and acetic acid remove serum proteins, iodolaminothanes and some organic iodine compounds. Further treatment of the resin with 50 percent acetic acid at a pH of 2 quantitatively removes T-4. Even though many modifications have been made in the original T-4 by column assay of Galton et al., the procedure is substantially the same i.e., a diluted serum sample is applied to an ion exchange resin. The unwanted contaminants are then eluted from the column and discarded. Following this, the hormones to be measured are eluted, collected and the T-4 determined by iodine analysis.

SUMMARY OF THE INVENTION

The present invention for determining T-4 is based on the competitive protein binding principle which is a modified form of saturation analysis. The T-4 to be determined is mixed with a determinant sample of T-4 labeled with a trace amount of radioactive isotope. A binding agent is added which will bind a definite number of molecules. Since the binding agent cannot distinguish between the labeled and unlabeled molecules, they compete with each other on an equal basis for the binding sites. These molecules are uniformly mixed so that the binding agent will bind them in the same ratio as that existing in the free or unbound state. As the concentration of the unlabeled molecules increases, the ratio becomes smaller and fewer labeled molecules are bound by the binding agent, leaving more of the labeled molecules in the free state. By calibrating the binding of labeled molecules in the presence of a known amount of unlabeled molecules, a quantitative procedure can be established.

In practicing the present invention, the crossedlink dextran gel column acts as the secondary binding agent for the unbound or free T-4, whereas the primary binding agent is a thyroxine (T-4) binding protein. First, a measured amount of serum is mixed with some T-4 labeled with radioactive iodine on top of the column. Both the column and T-4 mixture are at pH 12 to 13. At this pH, the T-4 binding serum proteins such as prealbumin, albumin and thyroxine binding globulin are completely dissociated from T-4. As the mixture flows down the column, the T-4 is bound by the dextran gel. The serum proteins are washed away with a barbitol buffer at a pH of 8.6 which automatically adjusts the pH of the column to that of the buffer. The pH is such that the transfer of the T-4 from the column to the primary binding agent is facilitated by the barbitol buffer. As the barbitol buffer is then added, Equilibrium is quickly established between the two binding agents and the primary binder is washed away carrying a portion of the T-4 with it. By measuring the amount of radioactivity on the column before and after treatment with the primary binding agent and comparing the percent retained by the column with a standard curve in which percent retained is plotted against T-4 concentration, the amount of T-4 in the serum can be determined.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention is predicated upon the discovery that a dextran gel column retains radioactive T-4 very well up to a pH of 9. Between pH 9 and 11, radioactive T-4 is rapidly eluted from the column whereas above pH 11 the retention of T-4 by the column is greatly enhanced. Thus, dextran gel binds T-4 but not protein, whereas anion exchange resins bind both T-4 and protein which are removed nonspecifically and only under acidic conditions.

The dextran gel employed in the column are crosslinked with various amounts of epichlorohydrin as described in U.S. Pat. No. 3,043,667 and have a water regain of about 5 grams per gram of dry gel product. Such gels are produced commercially by Pharmacia of Uppsala, Sweden and sold under the trade name of Sephadex in various molecular weight ranges and sieve sizes. Thus, Sephadex G-10 has a water regain of one gram per gram of dry gel, Sephadex G-15 has a water regain of 1.5 grams per gram of dry gel, Sephadex G-25 has a water regain of 2.5 grams per gram of dry gel and Sephadex G-50 has a water regain of 5 grams per gram of dry gel. Of these gels, Sephadex G-25 is preferred.

The dextran gel column employed in this invention is prepared by suspending 500 grams of dry gel in two liters of distilled water and allowing it to hydrate overnight. Fines are removed by straining the gel in 0.1 N sodium hydroxide for about 5 minutes, allowing it to settle for 15 minutes and then drying off the supernatant by suction. The process is repeated three times and the gel is suspended in 4,400 milliliters of 0.1 N sodium hydroxide. Four milliliters of this suspension is placed in a six milliliter plastic syringe barrel having a diameter of 13 millimeters and a length of 66 millimeters. Each barrel is prefilled with a bottom closure means, for example, a removable cap, and a detergent treated sintered polyethylene retaining disc about 1.5 millimeters thick and having a diameter of 13 millimeters is pressed coaxially to the bottom of the plastic barrel. After placement of the suspension in the syringe barrel, the suspension is stirred and permitted to settle free of air bubbles after which another detergent-treated sintered polyethylene disc, like the first-mentioned disc, is inserted into the syringe barrel and pushed
coaxially into firm contact with the gel. About 1.5 milliliters of sodium hydroxide solution remains above the upper disc. The upper end of the syringe is connected with a new polyethylene cap. This procedure provides a column containing about 450 milligrams of gel.

The T-4 test herein contemplated is performed by utilizing the gel column thus prepared as follows:

1. Remove the top cap from the column, decant the supernatant and place the column in an upright position.
2. Add 0.45 milliliter of a 0.1 N sodium hydroxide solution containing 0.10 microcuries of radioactive T-4 to provide from 60,000 to 120,000 counts per minute of gamma radioactivity.
3. Add 0.1 milliliter of serum sample and mix with the radioactive T-4. If a standard is to be determined, nonradioactive T-4 is added.
4. Remove the bottom cap and allow the serum mixed with the radioactive T-4 to flow into the column.
5. Wash the column with 4 milliliters of a 0.075 molar aqueous barbital solution buffered to a pH of 8.6.
6. Replace the bottom cap and determine the radioactivity of the column in counts per minute with a gamma counter.
7. Remove the bottom cap and add one milliliter of 0.15% human α-globulin dissolved in 0.075 molar barbital buffer.
8. Add 4.0 milliliters of 0.075 molar barbital buffer at pH 8.6 and allow to flow through the column.
9. Replace the bottom cap and again determine the radioactivity of the column.

10. Calculate the percentage of radioactivity retained on the column and determine the T-4 content of the serum sample by comparing the percent retention to a standard curve prepared with T-4 solutions of known concentrations.

Although a specific embodiment of the test method comprising this invention has been described, it should be understood that several variations are possible. Thus, the gel column can be made alkaline by potassium hydroxide or ammonium hydroxide if desired. Human α-globulin can be replaced with an equivalent amount of human serum, bovine serum or bovine gamma globulin as the primary binding agent. Aqueous alkaline solutions buffered to a pH of from 8 to 10 with sodium phosphate or tris (hydroxymethyl) amino methane can be used at concentrations from 0.01 to 0.2 molar, but an aqueous barbital solution is preferred, since it facilitates better quantitation when used to dissolve the human α-globulin or other binding agents.

Another variation of the present invention involves determining the radioactivity of the solutions before addition to the gel column and after elution therefrom rather than determining the radioactivity of the column itself. Percent retention is then determined by a difference calculation. However, for the sake of efficiency, it is preferable to determine the radioactivity of the column before and after elution.

Assays for T-4 by the column methods of the prior art should not be confused with the present method which involves saturation analysis using a radioactive tracer. Previously, a column was utilized only to separate contaminating iodines prior to analysis of iodine. In certain cases, this was done by using an ion exchange resin column at an alkaline pH, and iodine analysis was performed on the T-4 recovered from the column by measuring the effect of iodine on the ceric-arsenious acid reaction. The saturation analysis method herein disclosed is a direct determination of thyroxine, rather than the indirect measurement of thyroxine as iodine.

What is claimed is:

1. A process for the in vitro determination of thyroxine in serum comprising:
   A. adding a predetermined quantity of serum to be tested and a predetermined radioactive thyroxine solution to a column packed with a dextran gel crosslinked with epichlorohydrin and having a water regain of from 1 to 5 grams per gram of dry gel at a pH of at least about 11 and allowing the serum and thyroxine to flow into the column;
   B. washing the column with an aqueous alkaline solution;
   C. adding a predetermined quantity of an eluting agent containing thyroxine binding protein to partially remove the thyroxine on the column;
   D. determining the ratio of radioactive thyroxine retained by the column to that originally added, and
   E. calculating the thyroxine content of the serum by comparing the percent retention to that obtained with known concentrations of thyroxine in serum.

2. A process as in claim 1 wherein the radioactivity of the gel column is determined before and after elution to determine the ratio of radioactive thyroxine retained by the column to that originally added.

3. A process as in claim 1 wherein the aqueous alkaline solution is buffered to a pH of about 8 to 10.

4. A process as in claim 3 wherein the aqueous alkaline solution is a barbital buffer having a pH of 8.6.

5. A process as in claim 1 wherein the pH of the column is maintained at about 11 to 13.

6. A process as in claim 1 wherein the eluting agent is serum.

7. A process as in claim 1 wherein the eluting agent is human α-globulin.
UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,659,104 Dated April 25, 1972

Inventor(s) Jack Gross, Amirav Gordon and Lloyd Alan Schick

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 4 - Line 20

Third word should read "determinate" rather than "predetermine"

Signed and sealed this 29th day of August 1972.

(SEAL)
Attest:

EDWARD M. FLOTCHER, JR.
Attesting Officer

ROBERT GOTTSCALK
Commissioner of Patents
UNITED STATES PATENT OFFICE
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