

July 10, 1973

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METHOD FOR DETERMINING THYROXINE IN BLOOD SERUM
AND REAGENT THEREFOR

3,745,211

Filed April 8, 1971

3 Sheets-Sheet 1

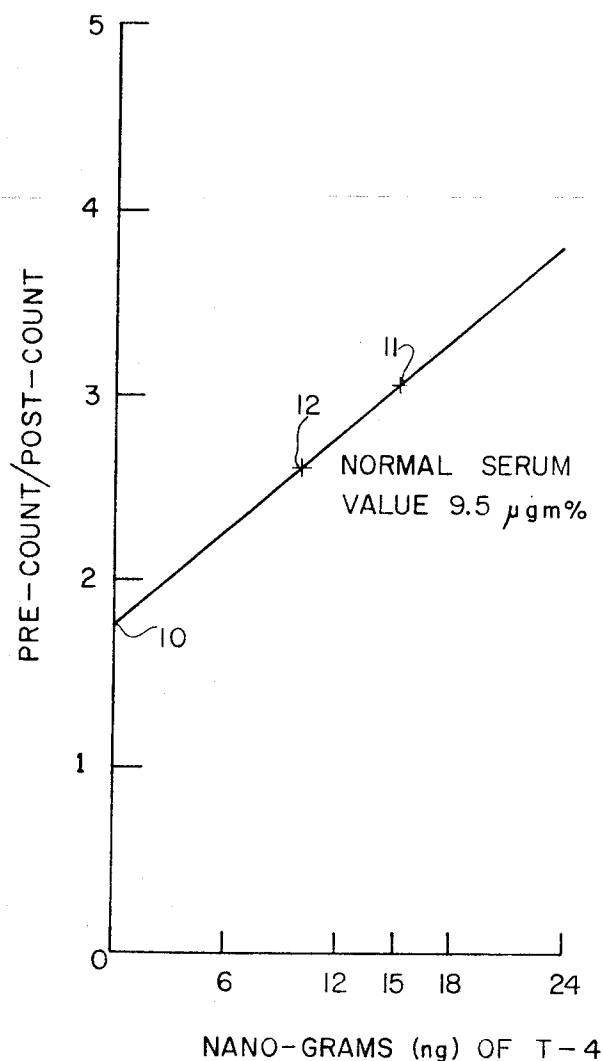


FIG.1

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3 Sheets-Sheet 2

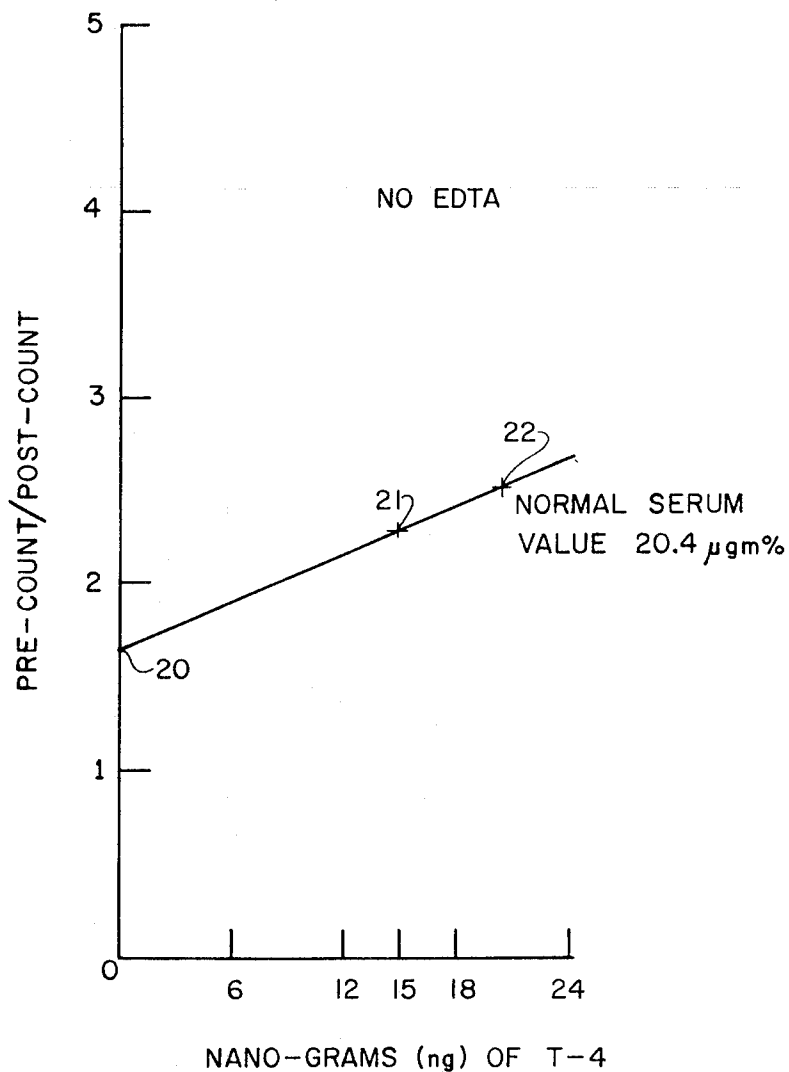


FIG. 2

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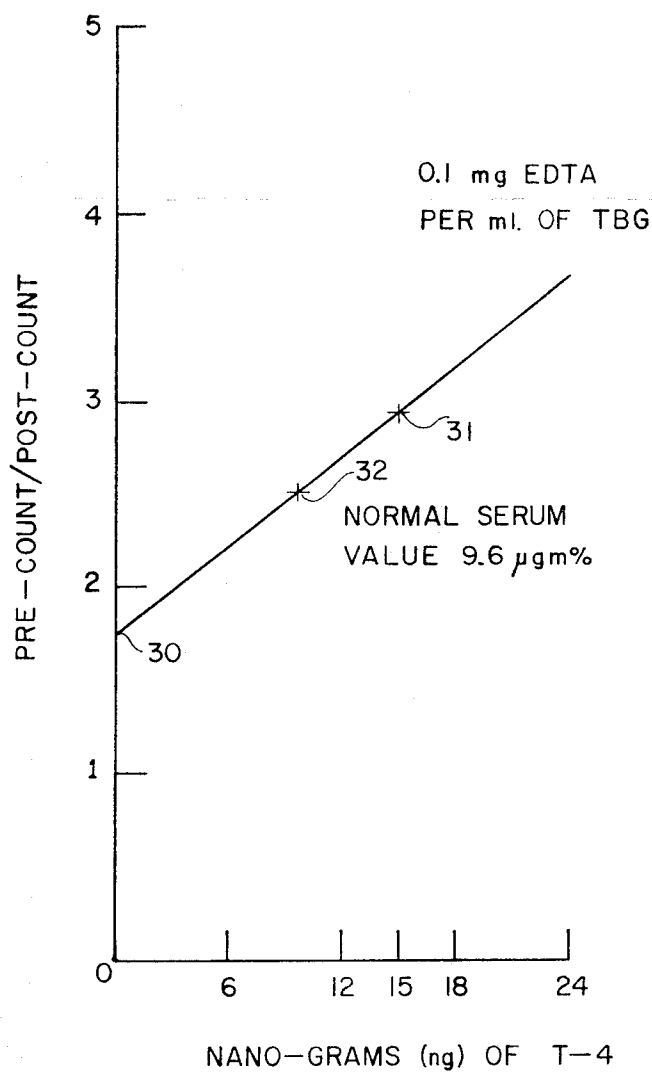


FIG.3

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METHOD FOR DETERMINING THYROXINE IN BLOOD SERUM AND REAGENT THEREFOR**James L. Brown and Floyd P. Hallett, St. Louis County, Mo., assignors to Mallinckrodt Chemical Works, St. Louis, Mo.**

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9 Claims

ABSTRACT OF THE DISCLOSURE

A buffered reagent comprising radioactive thyroxine and a chelating agent. The use of such a solution containing thyroxine-binding globulin in the radiometric serum thyroxine assay method of James L. Brown (application Ser. No. 865,785, filed Oct. 13, 1969) improves the reliability of the results obtained therefrom.

BACKGROUND OF THE INVENTION

The serum protein, thyroxine-binding globulin ("TBG") has a relatively high and specific affinity for binding the thyroxine hormone substance thyroxine (hereinafter referred to as "T-4"). It is also known that if radioactive T-4, that is T-4 containing radioactive iodine, such as iodine-125, is added to a solution containing barbital buffered TBG, essentially all of the T-4 will be bound to the TBG. If stable T-4 is then added to the TBG solution, the radioactive T-4 will be displaced from the TBG in proportion to the amount of stable T-4 that is added. If another T-4 binding agent, such as an ion-exchange resin, is now added to this system, it will bind the radioactive T-4 that has been displaced from the TBG.

Various techniques based on the above principles have been devised. For example, the degree of radioactive T-4 displacement and hence the amount of stable thyroxine added to the TBG can be determined by removing the ion-exchange resin with its bound radioactive T-4 from the system and comparing the radioactivity now emitted by the TBG solution with its original radioactivity. When increasing amounts of stable T-4 are added to solutions containing the same amounts of radioactive T-4 and TBG and treated as above, the radioactivity of the TBG solution decreases with each successive increase in added stable T-4. By plotting the ratio of radioactivity count before to the count after addition of stable T-4 vs. the amount of stable T-4 added, a straight line graph is obtained. If unknown amounts of stable T-4 are added to the TBG solution and treated in an identical manner, the amount of T-4 thus added can be read from the graph.

For this determination, diluted blood serum is customarily used as the source of TBG.

In order to determine T-4 in blood serum, the serum T-4 must first be separated from the binding proteins. This is accomplished by denaturing the proteins with alcohol. The denatured proteins release most (approximately 80%) of their bound T-4 which is removed in the alcoholic supernatant after centrifugation.

A particularly convenient method of determining serum T-4 is disclosed in the coassigned copending application of James L. Brown, Ser. No. 865,785, filed Oct. 13, 1969. Brown's method comprises the steps of adding to a known quantity of a reagent consisting essentially of a solution containing thyroxine-binding globulin and radioactive thyroxine, an alcoholic extract of a sample of blood serum whose thyroxine content is to be determined and an ion-exchange resin, maintaining the globulin solution in contact with the resin for a predetermined length of time, separating the ion-exchange resin from the globulin solution, and comparing the radioactivity of the globulin solution with that of globulin solutions to which have been

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added known amounts of stable thyroxine dissolved in the same volume of alcohol from the same source as that present in the said alcoholic serum extract.

Brown's method yields excellent results in the majority of cases. However, from time to time the use of Brown's method yields inaccurate and/or imprecise results; that is, erroneously high T-4 values are sometimes obtained, and the reproducibility of replicate determinations is sometimes unacceptable.

In the Brown method, the quantity of thyroxine in a blood sample is a linear function of the ratio between the radioactivity of the reagent/sample mixture before removal of the ion-exchange membrane and its radioactivity after removal of the membrane, as measured by a radioactivity counting device. This ratio, referred to herein-after as the pre-count/post-count ratio, is relatively high when the sample contains a large quantity of thyroxine since a relatively large quantity of radioactive thyroxine is thereby displaced from the globulin and removed from the solution by the ion-exchange resin. Conversely, when the quantity of thyroxine in the sample is low the pre-count to post-count ratio is also low. The problem noted above, which occasionally presents itself in the practice of Brown's method, is normally manifested as an abnormally low pre-count to post-count ratio for a standard serum sample containing a given quantity of thyroxine. This problem usually arises with standard solutions which are used in establishing the graphical relationship between the thyroxine content and pre-count to post-count ratio, on which also potentially arise with unknown samples on which determinations are to be made after the standard curve has been established. A continuing need has existed, therefore, for a practical means of improving the accuracy and reliability of the Brown method.

SUMMARY OF THE INVENTION

Among the several objects of the present invention may be noted the provision of an improved method for determining the thyroxine content of blood serum; the provision of such a method which possesses a high degree of accuracy and reliability; the provision of a special reagent which prevents certain measurement errors which have occasionally arisen in the use of previously disclosed methods; and the provision of a kit incorporating such reagent which facilitates the practice of the method of the invention. Other objects and features will be in part apparent and in part pointed out hereinafter.

The present invention is therefore directed to a reagent for use in the determination of the thyroxine content of blood serum which provides increased reliability in such determinations. This reagent comprises a buffered solution containing radioactive thyroxine and a chelating agent. The invention also includes such a composition which contains a blood serum from which most of the naturally occurring thyroxine has been extracted. The invention is further directed to an improvement in a method for determining the thyroxine content of blood serum using radioactive thyroxine as a tracer compound. This method comprises the steps of adding to a known quantity of a reagent consisting essentially of a solution containing thyroxine-binding globulin and radioactive thyroxine: (a) an alcoholic extract of a sample of blood serum whose thyroxine content is to be determined, and (b) an ion-exchange resin; maintaining the said globulin solution in contact with the said resin for a predetermined length of time; separating the ion-exchange resin from the globulin solution; and comparing the radioactivity of the globulin solution with that of globulin solutions to which have been added known amounts of stable thyroxine dissolved in the same volume of alcohol from the same source as that present in said alcoholic extract. The improvement comprises incorporating a chelating

agent into the solution which contains the thyroxine-binding globulin. The invention also includes an improved kit for facilitating the practice of the method.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a plot of serum T-4 values obtained by the method of Brown using a normal TBG/radioactive T-4 reagent;

FIG. 2 is a similar plot yielding erroneously high serum T-4 values for the same serum because of an abnormal TBG/radioactive T-4 reagent;

FIG. 3 is a similar plot yielding normal serum T-4 values for the same serum, using the TBG/radioactive T-4 reagent of FIG. 2, to which 0.1 mg. of the disodium salt of ethylenediaminetetraacetic acid per ml. of reagent had been added.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has now been discovered that, by using a reagent containing a chelating agent in addition to TBG and radioactive thyroxine, the occasional inaccuracies incurred in following the Brown method are avoided and a highly reliable method for determining blood thyroxine is provided.

Although we do not wish to be held to any particular theory, it is believed that the occasional inaccuracies which arise in the use of the Brown method may be due to catalytic decomposition of T-4 in the standard solution when it is mixed with the reagent, resulting from the presence of trace metallic ions such as cupric ion, ferric ion, etc., in the reagent solution. Decomposition of T-4 in the standard solution leaves less of the stable T-4 available to displace radioactive T-4 from the TBG during the mass transfer operation which takes place when the sample and reagent are mixed. Thus, a disproportionately low amount of radioactive T-4 is absorbed by the ion-exchange resin, the post-count measurement is disproportionately high, and the pre-count to post-count ratio is disproportionately low. When a chelating agent is incorporated in the reagent in accordance with the present invention, it avoids this problem, presumably by complexing ions such as cupric and ferric ions and thereby preventing these ions from causing catalytic decomposition of thyroxine.

For consistent results to be obtained, it has been found to be essential that the chelating agent be incorporated in the TBG reagent. The reason for this may be that ions such as cupric and ferric are initially present in the TBG reagent. Such ions are possibly introduced into the TBG extract as it passes through the ion-exchange column during removal of its naturally occurring thyroxine. In any event, we have found that the addition of a chelating agent to the standard solution does not satisfactorily prevent the aforesaid inaccuracies and only by addition of a chelating agent to the reagent has the high level of reliability of the present invention been achieved.

As illustrated in Example 3 below, the occasional inaccuracies in the Brown method have manifested themselves in abnormally low pre-count to post-count ratios for the standard solutions rather than for the unknowns. The reason for this is not fully understood, but it may relate to the fact that the standard solutions are not serum extracts whereas the unknown sample is such an extract and may contain native agents which protect it against metal ion-initiated thyroxine decomposition. It will be understood by those skilled in the art, however, that the provision of a chelating agent in the TBG reagent, in accordance with the present invention, protects against potential decomposition of the thyroxine introduced from either the standard solution or the unknown serum solution.

A wide variety of chelating agents may be used in the reagents and methods of the present invention and the

nature of the particular chelating agent employed is not critical. Particularly useful chelating agents are amino-polycarboxylic acids, such as ethylenediamine-tetraacetic acid (EDTA), and the salts thereof such as alkali metal salts. Other useful amino-polycarboxylic compounds include nitrilotriacetic acid (NTA), diethylenetriaminepentaacetic acid (DTPA), N-hydroxyethylenediamine-tetraacetic acid (HEDTA), 1,2-diaminocyclohexane tetraacetic acid and the respective salts of these acids. Additional exemplary chelating agents which may be used in the invention include deferoxamine, sodium diethyl dithiocarbamate, hydralazine, methisazone, 8-hydroxyquinoline, β -mercaptovaline, N-acetyl- β -mercaptovaline, 5-amino-1-phenyl 1H-tetrazole, isonicotinic acid hydrazide, 2,3-dimercapto-1-propanol and the other chelating agents disclosed in Scientific American, May 1966, pp. 40-50.

The novel reagent of the present invention is prepared using a solution containing thyroxine-binding globulin from which most of the naturally occurring thyroxine has been removed in accordance with the procedure described in the aforementioned application of James L. Brown. In one embodiment of the invention, a buffered solution of radioactive thyroxine and chelating agent is prepared first and the solution of TBG is added to it to produce the reagent used directly in serum analyses. In an alternative embodiment, the chelating agent is added to a buffered solution of TBG and radioactive thyroxine.

The following examples illustrate the practice of the invention.

EXAMPLE 1

To 78 ml. of serum is added 1850 ml. of a pH 8.6 barbital buffer solution containing 2.39% sodium barbital and 0.7% sodium azide (microbiological preservative), pH adjusted with HCl. A small amount of radioactive T-4 (approximately 0.13 mc.) is also added to serve as a tracer for determining the efficiency of the extraction.

An extraction column is prepared by adding a sufficient amount of an ion-exchange resin to a suitable glass column to provide a bed volume of 1,000 ml. and a flow rate of approximately 10 ml. per minute. For this purpose, a strongly basic quaternary ammonium type resin is suitable. For example, a commercially available resin of this kind designated "Amberlite IRA-400" (manufactured by the Rohm and Haas Company) is satisfactory, but other anion-selective or cation-selective resins may be used instead.

The resin is added to the column as a water slurry and a layer of water is always maintained above the resin. The column is connected to a reservoir containing the serum solution to be extracted, and flow of the serum through the column is begun. Periodically a 1 ml. sample of the eluate is removed and its radioactivity counted to measure the efficiency of the extraction. The radioactivity of the eluate should be 10% or less that of the stock solution.

To approximately 8 ml. of the above barbital buffer add sufficient (0.215 gm. EDTA- Na_2) disodium EDTA to provide a concentration of 0.1 mg. disodium EDTA per ml. of column eluate (extracted serum solution). Dissolution of the EDTA is effected with the aid of low heat. After cooling to room temperature, this solution is added to the column eluate (extracted serum solution). More radioactive thyroxine (approximately 0.27 mc.) is then added to the eluate. The extracted serum solution is then preferably passed through a 0.22 μ filter.

At this point, a volume (11,580 ml.) of barbital buffer solution containing 0.1 mg. disodium EDTA per ml. equal to 6 times the volume of the eluate is measured out. This solution is then added to the eluate providing a 6-fold dilution of the eluate. The resulting reagent has a pH of approximately 8.6 and a disodium EDTA concentration of 0.1 mg./ml.

EXAMPLE 2

About 10 ml. of blood whose thyroxine content is to

be determined is withdrawn and allowed to coagulate. The serum is removed and 1 ml. of the serum is added dropwise to 2 ml. of 95% ethanol in a centrifuge tube. The contents are then well mixed to denature the serum proteins. This alcoholic mixture is then centrifuged at 2500 r.p.m. for 4-5 minutes and 0.3 ml. of the supernatant liquid is withdrawn and transferred to a suitable test vial which contains 4 ml. of the TBG/radioactive T-4 reagent whose preparation is described in Example 1.

In the same manner, two standard samples are prepared. In place of the ethanolic serum extract, 0.3 ml. of an ethanolic solution containing a known amount (e.g., 0, and 12 nanograms) of stable thyroxine is added.

A strip of ion-exchange resin membrane is then added to each test vial. Since thyroxine is amphoteric, the ion-exchange resin may be either anion-selective or cation-selective. Many such resins are available in the form of membranes. For example, a commercially available anion-selective resin suitable for the purposes of this invention is designated "AR-111" (manufactured by Ionic Incorporated, Watertown, Mass.).

After the resin strips are added, the vials are capped and rotated for exactly 1 hour at room temperature. It is convenient to use one of the commercial rotators especially designed for this purpose. Since the resin uptake of radioactive thyroxine is a function of rotation time, it is essential that the rotation time be the same for both the unknown and control samples. At the end of the 1 hour rotation time, the resin strip is removed with forceps and discarded. The radioactivity of each vial is then counted and recorded as the "post-count." A minimum of 10,000 counts per minute will insure an error of less than 2%. The "pre-count" is determined by counting a similar vial containing only the 4 ml. of TBG/radioactive T-4 reagent. The pre-count/post-count ratio is calculated for each of the samples. The values for the standard samples are then plotted against the amount of added T-4, a straight line is drawn through the points, and the thyroxine content of the unknown serum samples is read from the resulting graph. Since the alcohol extracts approximately 80% of the T-4 from the serum, the value read from the graph should be divided by the extraction efficiency (approximately 0.8) to give the actual thyroxine content of the serum. This corrected value then represents the amount of T-4 (nanograms) in each 0.1 ml. of serum, which is numerically equivalent to the T-4 $\mu\text{gm.}$ percent of patient serum. One $\mu\text{gm.}$ percent is defined in the art as one $\mu\text{gm.}/100$ ml. serum. Since the serum sample is diluted with two volumes ethanol per volume serum and a 0.3 ml. ethanol/serum sample is tested the effective volume of serum tested is 0.1 ml. Thus, e.g., 0.1 ml. of a 12.0 $\mu\text{gm.}$ percent thyroxine serum contains

$$\frac{12.0 \mu\text{gm.}}{100 \text{ ml.}} \times \frac{0.1 \text{ ml.}}{\text{Sample}} = \frac{12.0 \times 10^{-3} \mu\text{gm.}}{\text{Sample}} = \frac{12.0 \text{ ng.}}{\text{Sample}}$$

EXAMPLE 3

The drawings illustrate the problems sometimes encountered with the above-discussed reagent of Brown and the prevention of these problems by use of the improved reagent of the invention.

The straight line graphs of FIGS. 1, 2, and 3 were each constructed by running two standard samples as outlined in Example 2. In each case, one standard was an ethanolic solution containing no thyroxine and the second standard was a similar solution containing exactly 12 nanograms of stable thyroxine. In each case, the pre-count/post-count ratio was determined and the three pairs of points were plotted to establish the three standard curves. In each case, the 12 ng. point was corrected for the 80% extraction efficiency and was therefore plotted as representing 15 ng. (12 ng./0.80=15 ng.). This enables one to read corrected serum T-4 values directly from the standard curve.

A portion of a single standardized normal serum sample (the "unknown" in this case) was carried through the pro-

cedure in parallel with the 0 and 12 ng. standards. The serum T-4 content of the standardized normal serum sample had been carefully and independently established as 9.3 $\mu\text{gm.}$ percent by averaging results obtained with standard Murphy-Pattee and Protein Bound Iodine (PBI) methods. The Murphy-Pattee method is described in the article by Murphy, B. E. P. and Pattee, C. J.: "Determination of Thyroxine Utilizing the Property of Protein Binding," J. of Clinical Endocrinology, 24:187, 1965. Numerous methods for determining PBI are known to the art, e.g., the methods described by Robbins, J. and Rall, J. E. "The Iodine-Containing Hormones," "Hormones in Blood," C. H. Gray and A. L. Bacharach, ed., vol. 1, 2d edition, Academic Press, New York, pp. 405-413.

In obtaining the data portrayed in the drawings, the 0 and 12 ng. thyroxine standards and the standardized normal serums were common to all three sets of determinations. The varying element among the three sets was the TBG/radioactive T-4 reagent. The radioactivity count data from which FIGS. 1, 2 and 3 were constructed is set forth in Table I.

TABLE I

Data for—	Sample	CPM		
		Pre-count	Post-count	Ratio
Figure 1....	0 ng. standard.....	44,060	23,849	1.84
	12 ng. standard.....	43,884	14,400	3.04
	Standard normal serum..	43,449	16,596	2.61
Figure 2....	0 ng. standard.....	30,910	18,668	1.65
	12 ng. standard.....	30,859	13,537	2.28
	Standard normal serum..	30,979	12,368	2.50
Figure 3....	0 ng. standard.....	30,677	17,386	1.76
	12 ng. standard.....	30,865	10,475	2.95
	Standard normal serum..	31,221	12,399	2.52

In the drawings, points 10, 20 and 30 represent the respective pre-count/post-count ratios for the 0 standards and points 11, 21 and 31 represent the respective readings for the 12ng. standards (corrected to 15 ng.). Points 12, 22 and 32 are the respective points plotted for the standardized normal serum sample.

In the determinations plotted in FIG. 1, a normal TBG/radioactive T-4 reagent as described by Brown (Lot Q) was used. The results were as expected.

In the determinations plotted in FIG. 2, another lot of TBG/radioactive T-4 reagent prepared as described by Brown (Lot S) was used. This resulted in an abnormal (low) pre-count/post-count ratio for the 12 ng. standard (Point 21), which, in turn, resulted in an erroneously high serum T-4 value (Point 22) for the standardized normal serum.

The determinations plotted in FIG. 3 utilized the defective reagent of FIG. 2 modified in accordance with the present invention by the addition of 0.1 mg. disodium EDTA/ml. This resulted in a normal value for the 12 ng. standard (Point 31) and a resulting accurate serum T-4 value for the standardized normal serum (Point 32).

The usefulness and accuracy of the method of the invention is increased if the analyst is provided with a kit containing all the special reagents and other supplies required. For example, a useful kit consists of a number of 4 ml. test vials made of glass and provided with screw caps. The vials should include 4 cc. of the TBG/radioactive thyroxine/chelating agent solution and should be matched for radioactivity. The standard samples should contain known amounts of stable thyroxine (e.g., 0 and 12 ng.) in 0.3 ml. of alcohol. A container of alcohol from the same source used to prepare the control samples should also be supplied. The kit should also include the required ion-exchange resin, the latter preferably in the form of membrane strips. The other necessary equipment such as syringes, radiation counter and rotator are standard laboratory equipment.

In view of the above, it will be seen that the several objects of the invention are achieved and other advantageous results attained.

As many changes could be made in the above methods and products without departing from the scope of the invention, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

What is claimed is:

1. A reagent for use in the determination of the thyroxine content of blood serum which provides increased reliability in such determinations, comprising a buffered solution containing radioactive thyroxine and a dissolved chelating agent for polyvalent metallic ions, said chelating agent being an amino-polycarboxylic acid or a salt thereof.

2. A reagent as set forth in claim 1 wherein the chelating agent is ethylenediaminetetraacetic acid or a salt thereof.

3. A reagent as set forth in claim 2 wherein the chelating agent is the disodium salt of ethylenediaminetetraacetic acid.

4. In a method for determining the thyroxine content of blood serum using radioactive thyroxine as a tracer compound, which comprises the steps of adding to a known quantity of a reagent consisting essentially of a solution containing thyroxine binding globulin and radioactive thyroxine, (a) an alcoholic extract of a sample of blood serum whose thyroxine content is to be determined, and (b) an ion-exchange resin; maintaining the said globulin solution in contact with the said resin for a predetermined length of time;

separating the ion-exchange resin from the globulin solution; and

comparing the radioactivity of the globulin solution with that of globulin solutions to which have been added known amounts of stable thyroxine dissolved in the same volume of alcohol from the same source as that present in said alcoholic serum extract, the improvement which comprises dissolving a chelating agent for polyvalent metallic ions in the solution

which contains the thyroxine-binding globulin, said chelating agent being an amino-polycarboxylic acid or a salt thereof.

5. A method set forth in claim 7 wherein the chelating agent is ethylenediaminetetraacetic acid or a salt thereof.

6. A method set forth in claim 5 wherein the chelating agent is the disodium salt of ethylenediaminetetraacetic acid.

7. A kit for determining the thyroxine content of blood plasma comprising a buffered solution of blood serum containing not more than about 20% of the normal amount of thyroxine, a dissolved chelating agent for polyvalent metallic ions, said chelating agent being an amino-polycarboxylic acid or a salt thereof, and a sufficient amount of radioactive thyroxine to act as a tracer;

two control samples containing a known amount of thyroxine dissolved in a predetermined volume of alcohol; a quantity of alcohol from the same source used to prepare the control sample; and a plurality of resin membrane strips whose dimensions permit them to fit loosely in portable test containers.

8. A kit as defined by claim 7 wherein the buffered solution contains a barbitol buffer.

9. A kit as defined by claim 7 wherein the buffered solution contains sodium azide as a preservative.

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