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METHOD OF DETERMINING THYROXIN IODINE IN BLOOD SERUM

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ABSTRACT OF THE DISCLOSURE

Method of determining the amount of thyroxin iodine in blood serum by first extracting thyroxin in an alcohol-liquid hydrocarbon solvent mixture and an aqueous buffer of citric acid-disodium phosphate. The thyroxin remains in the solvent mixture and after centrifuging, a portion of the supernatant liquid is treated with an aqueous alkaline organic reagent having bromate and bromide ions. With the addition of a mineral acid, bromine then liberates the iodine from its organic combination. After centrifuging again, the amount of iodine liberated is measured colorimetrically from a portion of the supernatant liquid. A sequestrant can be included with the buffer solution.

This invention relates to a method for the analytical determination of iodine in blood serum. The importance of this determination is due to the fact that iodine is an essential component of the thyroid hormone, thyroxin, which regulates bodily metabolism, and since the blood serum level of this hormone varies in diseases of the thyroid gland it is important for the physician to know that this level is.

The concentration of the thyroid hormone in the serum is so minute that no direct analytical method for its analysis is available and all methods for its estimation are based upon an analysis of the serum iodine and the hormone calculated from this iodine value.

The validity of this analysis depends upon the absence of other iodine compounds in the serum and this is generally (but not always) the case. Thus certain iodine compounds used for medication as well as some naturally occurring nonhormonal iodine compounds such as diiodotyrosine and monoiodotyrosine may be present and the analysis is then no longer valid for hormone concentration. Various procedures attempt to separate the thyroxin iodine from other forms of iodine but are only partially successful.

The most commonly used procedures for this purpose are the protein-bound iodine (PBI) (see Barker, S. B., Humphrey, M. J., and M. H. Soley, J. Clin. Invest., vol. 30, p. 55, 1951) and the butanol-extractable iodine (BEI) (see Chaney, A. L., Anal. Chem., vol. 12, p. 179, 1940). The PBI procedure is the most popular. In this method the blood serum proteins are precipitated with a protein precipitant and collected by centrifugation, and then washed several times. This removes any inorganic iodine that might be present but does not remove organic iodine compounds if present or iodinated protein which may form from inorganic iodine if present. The separated serum proteins must then be ashed, either by heating in a muffle furnace at high temperature, or by boiling with strong oxidizing acids. In the latter case the iodine must be separated from the strong acids by distillation in a special type of still.

The iodine is measured by its catalytic activity on causing the reduction of yellow ceric salts by arsenious acid. The yellow cerium is either decolorized or reduced in intensity as a result of the iodine and this change in color is proportional to the amount of iodine present. When

measured in a suitable colorimeter or spectrophotometer in comparison with a standard iodine solution, the concentration of iodine in the unknown sample can be calculated by means of standard formulae. This reaction is basic to all procedures used for the measurement of the serum hormonal iodine in the minute concentrations found in serum.

The second procedure most commonly used for the determination of the thyroxin iodine is called the butanol-extractable iodine (BEI) method. (See Man, E. B., Kydd, D. M., and J. P. Peters, J. Clin. Invest., vol. 30, p. 531, 1951.) In this method the iodine is extracted from the serum with the immiscible organic solvent, N-butanol. Because of the properties of this solvent, several extractions, usually 3 to 4, are required in order to quantitatively extract the thyroxin iodine from the serum. Any other iodine compounds that may be present are also extracted and are removed by washing the n-butanol extract three or four times with concentrated alkali. Thereafter, the butanol must be removed by evaporation, preferably in vacuo. The residue is then ashed in the same manner as the PBI and the ceric-arsenite reaction carried out as for the PBI. While the BEI is more specific than the PBI for thyroxin iodine, it is but little used as compared to the PBI. A specially trained analyst and a much greater amount of time are required.

The purpose of the invention described herein is to provide a procedure which will permit the analysis of blood serum for its thyroxin iodine content in a manner that is as specific as the BEI method and yet is at once simpler than either the PBI or BEI methods. This is made possible by the development of new and novel reagents and their manner of usage.

The combination of Reagent A and Reagent B, the extracting solvent, makes possible the quantitative extraction of thyroxin iodine in the organic supernatant liquid with but a single extraction, as compared to the 3 or 4 extractions heretofore used. Also, this combination of reagents is so designed that iodine contaminants that must be removed by washing with strong alkali in the conventional methods are not present and the method is thus further simplified by eliminating this washing.

Reagent C and Reagent H have been designed to permit the quantitative extraction of the thyroxin iodine from the organic solvent phase to an aqueous phase which is necessary before the colorimetric reaction can be carried out. Furthermore, this combination of reagents and extractions results in a final solution sufficiently pure as not to require ashing of the sample.

Reagent C contains bromide-bromate salts which on acidification with strong mineral acid result in bromine being released when bromide is oxidized by bromate. The bromine in turn liberates the iodine from its organic combination in the thyroxin molecule in such a manner that it reacts in the same manner with the ceric-arsenite color reagents as the inorganic iodine which requires ashing in the other procedures.

The saving of time by this procedure as compared to those presently used is estimated at about 50%. Furthermore, because of its simplicity specially trained analysts are not required and because of the reduced number of manipulations, a considerable gain in accuracy results.

The following list sets forth the combination of reagents used in the method described herein:

Reagent A: Sodium phosphate-citrate buffer. In a 1 liter volumetric flask, place 10 grams of sodium phosphate dibasic anhydrous (B&A), 6.6 grams citric acid monohydrate, 0.35 gm. ethylenediamine tetra-acetate (EDTA). Dissolve and dilute to mark with distilled water. Keep in refrigerator.

Reagent B: Butanol alcoholic mixture. To 700 ml. of

3-methyl-1 butanol (isoamyl alcohol) add 300 ml. of trimethylpentane.

Reagent C: Sodium hydroxide-sodium sulfate-sodium bromide-potassium bromate reagent. In a 1 liter volumetric flask place 50 grams of anhydrous sodium sulfate, 8 grams sodium hydroxide, 1.3 grams sodium bromide and 0.275 gram potassium bromate. Dissolve in distilled water and dilute to the 1 liter mark. The ratio of the bromide salt to the bromate salt is 3–7:1 by weight, respectively.

Reagent D: Perchloric acid, 3.2 N. Dilute 280 ml. of 70–72% perchloric acid to 1 liter with distilled water. Determine the concentration of acid by titration with standard NaOH using phenolphthalein as an indicator and adjust to the required normality.

Reagent E: Arsenious acid solution. In a 1 liter Florence flask place 5 grams of arsenic trioxide and 0.6 gm. sodium hydroxide. Add about 400 ml. of distilled water and boil until the arsenic trioxide has dissolved. Cool to room temperature, transfer to a 500 ml. volumetric flask and dilute to the mark with distilled water.

Reagent F: Ceric ammonium sulfate solution. In a 5-liter Florence flask place 73 grams of ceric ammonium sulfate. Add 400 ml. of distilled water and 600 ml. of concentrated sulfuric acid. Shake vigorously for several minutes, then add an additional 2000 ml. of distilled water and shake until the ceric ammonium sulfate has dissolved. Cool to room temperature and dilute to 4000 ml. with distilled water.

Reagent G: Thyroxin standards. Dry L-thyroxin to constant weight in a desiccator. Place 92 mgs. of this thyroxin in a 1-liter volumetric flask. Add 20 ml. of 1 N sodium hydroxide and shake until the thyroxin has dissolved. Add about 800 ml. of distilled water and 1 gm. of potassium sorbate. Dilute to the mark with distilled water. Accurately measure 1 ml. portions of this thyroxin standard into 16 x 125 screw-capped test tubes and close with a Teflon-lined screw cap. Preserve by placing in the freezing compartment of a refrigerator. Stability 6 months if kept frozen. Prepare a working standard daily by adding 9 ml. of distilled water to one of the tubes containing 1 ml. of the thyroxin standard. Dilute 1 ml. of this solution to 100 ml. with 0.01 N sodium hydroxide. This is the working standard and is discarded at the end of the day.

Reagent H: Dichloromethane (methylene chloride).

EXAMPLE

The procedure in performing the improved method of the determination of serum hormonal iodine utilizing the afore-mentioned reagents was performed as follows:

Step 1: Place 1 ml. of serum in a screw-capped test tube, 16 x 150 mm. In a similar tube place 1 ml. of distilled water for a blank, and another similar tube 1 ml. of the working thyroxin standard. To each tube add 2 ml. of the citrate buffer (Reagent A) and 4 ml. of the alcohol-solvent mixture (Reagent B). Cap and shake vigorously for three minutes, then centrifuge 3 minutes at 3000 r.p.m.

Step 2: Transfer a 2 ml. aliquot of the upper supernatant layer to a 16 x 125 mm. screw-capped test tube. Add 2 ml. of the sodium sulfate-sodium hydroxide, etc., solution (Reagent C), and 3 ml. of dichloromethane (Reagent H). Cap and shake vigorously for 2 minutes, then centrifuge 3 minutes at 3000 r.p.m.

Step 3: Transfer a 1 ml. aliquot of the supernate to a 19 x 150 mm. Coleman cuvette. To each tube add 5 ml. of 3.2 N perchloric acid (Reagent D). Place in a constant temperature water bath, at 30 degrees C. and allow to remain 10 minutes. Add to each tube, 1 ml. of the arsenious acid solution (Reagent E) and allow an additional 5 minutes for temperature equilibrium.

Step 4: At intervals of 30 seconds and in sequence add 1 ml. of ceric ammonium sulfate solution (Reagent F). After each addition the tube is returned to the water bath.

Step 5: Set the Coleman spectrometer to 100% transmittancy at about a wave length of 4300 Angstrom units with the machine empty. At the end of about 28 minutes place the blank in the instrument and adjust the wave length scale until the transmittancy of the blank reads 20. Remove blank and readjust transmittance to read 100 and, if necessary, readjust blank to 20. At the end of 30 minutes the blank should read 20% transmittancy with the instrument set to 100% transmittancy empty. Now read the standard and the unknowns at 30 second intervals in the same sequence used for addition of the cerium.

Step 6: Calculation

$$\frac{6 \times Uk}{Sk} = \text{megs. iodine per 100 ml.}$$

U=Transmittancy reading of the unknown (serum)

S=Transmittancy reading of the standard

k represents a value obtained from a table prepared by Robert H. Hamilton, Clinical Chemistry, vol. 8, p. 194, 1962, which gives a value that represents a straight-line relationship between the amount of color developed by the reagents with iodine and the amount of iodine present. This straight-line relationship is limited. If transmittancy reading is more than 80, a straight-line relationship no longer obtains and it is necessary to repeat the determination using a lesser amount of serum.

Having described my invention, I claim a unique combination of reagents and extraction procedure for the analysis of thyroxin iodine in blood serum in a manner that eliminates the necessity for ashing with a saving of time, a simplification of technique, and due to the lessened handling, an increase of accuracy. Almost all methods for determining the minute amounts of iodine present in blood are based upon the catalysis of the reduction of ceric ion by arsenious acid, the catalytic agent being iodine. The cerium is yellow, and in the presence of iodine, arsenious acid decolorizes the cerium, the extent and speed of the decolorization is proportional to the amount of iodine present. By measuring this color change in a spectrophotometer or colorimeter, the amount of iodine may be determined by comparison with a standard similarly treated.

However, this reaction is not very specific for iodine and it is necessary to remove interfering substances. In the case of protein-bound iodine, the most common procedure, the proteins are precipitated, collected by centrifugation, and then ashed, after which the cerium arsenic color reaction is performed. If a subject is receiving iodine medication, then this method cannot be used as the iodine from the medication will also be present.

In the present invention many of the steps proscribed in the prior art are eliminated. The alcohol-solvent mixture used for the extraction has been so devised that the thyroxin is quantitatively extracted with but a single extraction and iodides from medication or the naturally occurring nonthyroxin iodine compounds present in most serums are not extracted. Evaporation of the alcohol is not necessary since thyroxin is re-extracted into the aqueous phase by Reagent C, and when so extracted is sufficiently free from interfering substances that it does not need to be ashed. The reaction is further made possible by the release of bromine from Reagent C when perchloric acid, Reagent D, is added. The bromine splits the iodine from its organic combination in the thyroxine molecule and further enhances the sensitivity and accuracy of the reaction.

While a detailed description of the invention has been set forth in the described method for purpose of illustration, it will be apparent to those skilled in the art that many modifications may be made without departing from the principles of the invention. Thus the purpose of Reagent A is to provide the proper pH to facilitate extraction of the thyroxin. The composition of this buffer may be

varied in many ways to produce the same result and a sequestrant is preferred.

Within limits, the proportions of the components of Reagent B may be varied and other hydrocarbons, preferably petroleum hydrocarbons, such as hexane, heptane, etc. may be substituted for the trimethylpentane. Isoamyl alcohol is preferred, but other alcohols such as isobutyl are operative.

3.2 N perchloric acid is the mineral acid cited in the example given and is the preferred acid, but sulfuric acid of approximately 8 N strength can also be used.

In the bromide-bromate alkaline solution, the amount of bromate should be sufficient to oxidize substantially all of the bromide to bromine once acidification is effected.

Similarly, adjustments in the strength of the other reagents can be made without affecting the principle of the method as disclosed in the specification and claims.

I claim:

1. The method of determining the thyroxin iodine content in a serum sample comprising the steps of separating thyroxin by agitating said sample in a mixture of an aqueous dibasic phosphate-critric acid buffer solution wherein the molarity of the former is about 0.07 and of the latter is about 0.03, and an alcohol-liquid hydrocarbon solvent mixture wherein said alcohol is selected from the group consisting of isoamyl and isobutyl alcohol, said alcohol and said hydrocarbon being present in the ratio of about 5–2:1 by volume, respectively, centrifuging the agitated mixture, transferring an aliquot of the supernatant organic liquid to a separate container, adding an aqueous alkaline reagent containing a soluble bromide salt and a soluble bromate salt wherein the ratio of the former to the latter is 3–7:1 by weight respectively, centrifuging the resultant liquid and transferring an aliquot of the aqueous supernatant to a further container, adding sufficient mineral acid to release bromine which liberates substantially all iodine from the thyroxin, and thereafter determining the amount of iodine colorimetrically.

2. The method of claim 1 wherein the alcohol in the alcohol-solvent mixture is isoamyl alcohol and the solvent is trimethylpentane in amounts of about 5–2:1 by volume, respectively.

3. The method of claim 1 wherein the buffer solution contains a sequestrant, EDTA.

4. The method of claim 1 wherein the bromide and bromate are sodium bromide and potassium bromate.

5. A mixture for separating thyroxin from other iodine-containing substances in serum, said mixture comprising an aqueous dibasic phosphate-critic acid buffer solution wherein the molarity of the former is about 0.07 and of the latter is about 0.03 and an alcohol-hydrocarbon solvent mixture, said alcohol being isoamyl alcohol and said solvent being trimethylpentane in amounts of about 5–2:1 by volume, respectively.

6. An aqueous alkaline reagent for liberating iodine from thyroxin, said reagent containing a soluble bromide salt and a soluble bromate salt, the ratio of the former to the latter being 3–7:1 by weight, respectively.

References Cited

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