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METHOD FOR DETERMINING TOTAL BLOOD SERUM IRON-BINDING CAPACITY

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2 Claims

ABSTRACT OF THE DISCLOSURE

A method for determining the total iron-binding capacity of blood serum. The method comprises first releasing the iron normally present on the serum and known as the serum iron (SI). The free iron is then removed to prevent it from recombining with the serum. Next, the serum is saturated with a tracer amount of radioactive iron, an ion-exchange resin is mixed with the serum-radioactive iron mixture and the combined mixture is incubated. After incubation, the initial radioactivity of the mixture and ion-exchange resin is measured with suitable detecting means, after which all of the liquid is removed. The ion-exchange resin is then washed and the residual radioactivity determined. By suitable calculation, the total iron-binding capacity of the serum is then determined.

BACKGROUND OF THE INVENTION

Iron circulating in the plasma is known to be bound to the β_1 globulin fraction called transferrin. Transferrin has a definite total iron-binding capacity (TIBC) and is partially saturated by the iron, normally about one-third saturated. This quantity of iron is known as serum iron (SI). The unsaturated (latent) iron-binding capacity (LIBC) plus the serum iron (SI) is equal to total iron-binding capacity (TIBC). There are several tests known for the measurement of total iron-binding capacity involving a variety of techniques. Most widely used of the methods for determining iron in blood serum are those involving color reactions of ferrous iron with various reagents. Direct photometric methods make use of the fact that the iron complex is colored and may be directly estimated photometrically. Because the change in optical density is small, these methods are not entirely satisfactory. They are effected by turbidity and hemolysis and can produce erratic results. Visible hemolysis for example, causes high results. Some methods precipitate the protein in order to extract the iron from the serum proteins.

Measurement of iron concentration circulating in serum is useful in establishing a diagnosis of many diseases including iron deficiency, siderosis, or hemochromatosis and is helpful in determining the treatment of these and other disorders.

SUMMARY OF THE INVENTION

The method of the present invention utilizes an ion-exchange resin, preferably in a sponge form, and radioactive iron. To determine the total iron-binding capacity of serum using radioactive iron, essentially all of the iron on the serum protein must be removed. There are several known methods for releasing serum iron bound to protein by lowering the pH with an acid. All of these methods, however, lower the pH for the purpose of recovering the iron that is released and not for recovering the protein which is bound to the iron. In the present method, a lesser amount of acid is added, only enough to drop the serum pH to about 3.5 (± 0.5). This pH is low enough to release the serum iron but does not denature the transferrin. The free iron is then absorbed with an absorbent such as calcium carbonate and removed by centrifugation which pre-

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vents the free iron from recombining with the serum. The pH is then returned to about 7.5 with tris(hydroxymethyl)amino methane (tris). The result is a specimen of biologically active serum with transferrin capable of binding with iron but lacking the endogenous serum iron present in fresh serum. This serum is saturated with radioactive iron and the amount of iron binding to the serum is then determined.

The ion-exchange resin acts as the secondary binding site for radioactive iron which is added to the serum. When the radioactive iron is added to the serum, the transferrin rapidly reacts with the iron. The serum iron having been removed, the amount of iron which reacts with the protein represents the total iron-binding capacity of the serum. When the ion-exchange resin is added to the serum and radioactive iron solution, the excess iron which is not reacted with the transferrin binds to the resin. The total radioactivity added to the serum is counted in a suitable scintillation counter. After a suitable incubation period, the serum-radioactive iron solution is discarded and the resin is washed with iron-free water or other suitable washing preparation. The radioactivity remaining on the resin is then determined. This quantity represents the excess iron. The total iron-binding capacity can then be determined from the relationship

$$TIBC = \left(1 - \frac{S \times t}{T}\right) \times F \times 2 \times a$$

wherein S is the residual radioactivity remaining on the ion-exchange resin expressed in counts-per-minute; T is the initial or total radioactivity added to the serum; F is the total micrograms of ferric ion added; t is the TIBC correction factor; and a is an adsorbent correction factor. The correction factors are determined as hereinafter described.

DETERMINATION OF CORRECTION FACTORS

Prior to determining the total iron-binding capacity of serum, the effect of the different materials employed should be considered and taken into account in the calculations. For each lot of ion-exchange resin, radioactive iron and adsorbent used, a correction factor should be determined. These factors, which may be termed latent iron-binding capacity (LIBC), resin uptake (RU), adsorbent, and total iron-binding capacity (TIBC) correction factors, can be determined as follows:

LIBC

To one milliliter (1 ml.) of serum is added 10 micrograms (10 μ g.) of ferric chloride to saturate the serum with iron. Radioactive iron solution is then added, followed by addition of the ion-exchange resin. The combined serum, resin and iron solution is then incubated for 60 minutes at 45° C. The total radioactivity of the serum, resin and iron solution is determined and the serum-iron solution is then decanted and the resin washed with iron-free distilled or deionized water. The radioactivity remaining on the resin is then determined. A correction for background is taken into account in all readings, if necessary. The correction factor is then calculated by dividing the initial or total activity by the residual activity remaining on the resin. A typical correction factor is 1.22.

RESIN UPTAKE

Radioactive iron solution is added to the ion-exchange resin and the initial or total activity is measured. After incubation for 60 minutes at 45° C., the radioactive iron solution is decanted and the resin is thoroughly washed with iron-free water. The residual radioactivity remaining on the resin is then measured and the resin uptake calculated by dividing the initial activity by the residual activity.

A typical correction factor is 1.05. This correction factor is necessary because the resin normally takes up only about 95% of the iron, resulting in a correction factor of 1/.95 or 1.05.

TIBC

The total iron-binding capacity correction factor is calculated by subtracting the resin uptake factor (RU) from the latent iron-binding capacity (LIBC) correction factor and adding half the difference to the resin uptake factor. A typical calculation is as follows:

$$\text{corr. factor} = \frac{\text{TIBC} - \text{RU}}{2} + \text{RU} = \frac{1.22 - 1.05}{2} + 1.05 = 1.135$$

ADSORBENT CORRECTION FACTOR

Since not all of the adsorbent is removed, a correction factor is determined as follows: hydrochloric acid is added to the adsorbent and allowed to react for about 5 minutes. A volume of tris(hydroxymethyl)amino methane (tris) equal to the volume of hydrochloric acid is added to the mixture, which is then mixed and centrifuged to obtain a clear supernatant solution and a precipitated portion. 1 ml. of radioactive iron solution is added to 1 ml. of a 50:50 mixture of the clear solution and water. The ion-exchange resin is added and the combined resin-solution is incubated for 60 minutes at 45° C. The initial or total activity is measured; the solution is decanted and the resin washed with iron-free water after which the residual activity remaining on the resin is measured. The correction factor for the adsorbent is then calculated by dividing the residual activity by the initial activity to obtain a percentage figure, typically 0.90 or 90%.

DETAILED DESCRIPTION

Preparation of radioactive iron solution

The tracer amount of radioactive iron, Fe⁵⁹, can be prepared from suitable sources by proper dilution. While any ferric iron will bind to blood serum, ferric ammonium citrate has been found to be particularly suitable since it can be buffered to a final pH of about 7.0. The iron-serum protein complex partially dissociates when the pH is below 7, and dissociates more vigorously when the pH is below 6. Ferric chloride, for example, requires a low pH in order to maintain the ferric ion in solution, and is therefore not as desirable. The amount of activity should be a workable amount for tracer purposes. This will depend on, among other factors, the amount of blood serum used and the sensitivity of the recording and detecting instruments. This requirement is readily understood by the skilled workers in the art. For the purposes of the present invention, an activity of 0.1 microcurie or less of Fe⁵⁹ per milliliter of solution has been found to be desirable. Since the unsaturated iron-binding capacity of normal serum is about 2.5 micrograms per milliliter (2.5 μg./ml.), a desirable ferric iron concentration in the final solution has been found to be in the range of about 5-7 μg./ml. although other concentrations are suitable. The source of radioactive iron, Fe⁵⁹, is diluted with iron-free water so that the concentration of ferric ion is about 12 μg./ml. and the activity is less than 0.2 μc./ml. To this solution is added an equal amount, by volume, of buffer which comprises a solution of sodium chloride, sodium barbiturate, and diethyl barbituric acid dissolved in iron-free water. Each milliliter of final solution will then contain about 6 μg. of iron and less than 0.1 μc. of activity. The pH should be about 7.0-7.5.

The resin sponge employed in this invention comprises a polyurethane foam of intercommunicating cell type containing a strong base anion-exchange resin as described in U.S. Pats. 3,024,207 and 3,094,494. Such a urethane foam resin may be prepared by incorporating the ion-exchange resin particles in a mixture of a polyether or polyester and a polyisocyanate and then sub-

jecting the mixture to the usual conditions for producing foams of the polyurethane type. An example of a suitable ion-exchange resin is a strongly basic anion-exchange resin chloride form, such as that marketed under the trademark Amberlite IRA400. Such resins can be prepared by the process disclosed in U.S. Pat. 2,591,573. The resin which is utilized should not remove iron already bound to the globulin molecule nor bind the whole molecule.

The resin sponge can be made in various forms. A convenient embodiment is a cylindrical plug which can be easily placed in the bottom of a container adapted for placement in the well of conventional scintillation counters. The actual dimensions of the cylindrical plug of resin sponge will be determined by the volume of serum employed in the test and by the size of the container utilized to hold the serum and the radioactive solution. It is desirable that a plug of standard size and a standard volume of serum be established in utilizing the method of the present invention. Various modifications can be made in the type of resin and the content thereof in the polyurethane foam or in the makeup and characteristics of the sponge. Modifications can also be made in the volume of serum and amount of tracer material employed in the method. Such variations will not detract from the operability of the method. To obtain the greatest advantage from the practice of the method, a selected volume of serum and a selected size of a particular resin sponge should be adapted as standards.

The following examples are illustrative of the present invention and are presented to illustrate the practice of the method.

EXAMPLE I

A solution of ferric ammonium citrate is made from Fe⁵⁹Cl₃. Ferric chloride Fe⁵⁹ solution of approximately 300 microcuries activity is added to a 1000 ml. volumetric flask. 52.3 mg. of anhydrous ferric chloride is added to the flask. This represents 18 mg. of iron. Six drops of concentrated ammonium hydroxide is added to form a precipitate of ferric ammonium hydroxide Fe⁵⁹. Just enough citric acid crystals, about 2.1 gms., are added to dissolve the precipitate. The flask is agitated and approximately 500 ml. of iron-free water is added. Dilute (1:10) NH₄OH is added until a pH of 7.0 is reached. The solution is then diluted with iron-free water to a total of 1000 ml. After an iron assay, the solution is further carefully diluted to provide 12 μg./ml. of iron and an activity of approximately 0.20 μc./ml. The addition of benzyl alcohol in a concentration of 0.9% does not interfere with the effectiveness of the test.

Buffer solution is made by dissolving 6.4 gms. of sodium chloride and 2.3 gms. of sodium barbiturate in 500 ml. of iron-free water, 6.0 gms. of diethylbarbituric acid is added, dissolved, and the solution is then diluted to 1000 ml.

The ferric ammonium citrate Fe⁵⁹ solution is mixed with the buffer solution in a one-to-one ratio so that each 1.0 ml. of final solution contains about 6 μg. of iron and less than 0.1 μc. of activity.

EXAMPLE II

A container suitable for seating in the well of a scintillation counter is used to receive a polyurethane foam-anion resin sponge. The sponge is cylindrical in shape and has a diameter of 7/16 inch and a length of 1 1/16 inch. About 5 ml. of venous blood is withdrawn from a subject and placed in a separate test tube. The blood is permitted to stand to allow clotting and then centrifuged to separate the serum. The serum is removed and 1 ml. is pipetted into an iron-free tube containing 0.5 ml. of 1 N HCl. Any acid can replace the HCl provided the pH is low enough to liberate iron from serum protein, but not so low as to denature the iron-binding protein. The serum and hydrochloric acid is mixed and incubated at room temperature (23° C.) for five minutes. The released iron is adsorbed

by adding to the serum-acid mixture about ½ gram of powdered calcium carbonate and mixing gently for five minutes. The CaCO₃ is used in excess, consequently the ½ g. amount is not critical. Any material which will bind the free iron can be used as the adsorbent, i.e., ion-exchange resins or alkaline earth insoluble compounds for example. CaCO₃ is used because of its low solubility in water, its buffering effect and because it does not bind serum protein. The pH is then returned to the physiological range by adding 0.5 ml. of 0.2 N trishydroxymethylamino methane (tris) and mixing. The amount and concentration of trishydroxymethylamino methane (tris) which is used is important. The pH must be returned to about 7.5 for best results and to 7-8 for acceptable results. While the use of tris is preferred since it provides a buffering action making it easier to control the pH; other basic compounds can also be used. The released iron which is now adsorbed onto the calcium carbonate, thereby preventing it from recombining with the serum, is removed together with the calcium carbonate by centrifuging the combined mixture. 1 ml. of the clear supernatant is withdrawn and added to a tube containing the ferric⁵⁹ ammonium citrate-buffer solution previously described. This provides a tracer amount (less than 0.1 μc.) of radioactive iron and a concentration of about 6 μg. of iron for saturation of the iron-free serum. The combined serum and ferric⁵⁹ ammonium citrate is allowed to incubate for about 10 minutes. Although binding of the iron to the serum protein is rapid, the 10-minute incubation period assures complete binding.

The ion-exchange resin sponge is then added to the tube and the combined serum, resin sponge and ferric⁵⁹ ammonium citrate solution are allowed to incubate for one hour at 45° C. The one-hour incubation period was found to be sufficiently long, but longer periods do not introduce any error. During the one-hour incubation period, the tube is placed in the well of a scintillation counter so that the well surrounds the resin sponge within the tube. The radioactivity counts are recorded and correction is made for any background activity. The recorded value represents the initial radioactivity of the combined serum, ferric⁵⁹ ammonium citrate solution and the resin sponge.

The tube is then removed from the counter and, after completion of the incubation period, all of the liquid is aspirated from the tube. The resin sponge is then washed several times with two or three milliliters of iron-free distilled or deionized water after which the tube is returned to the well of the scintillation counter. The radioactivity remaining on the sponge is then determined with correction for any background as in the previous reading. The recorded value represents the residual activity remaining on the resin sponge after the foregoing washing steps. The percent figure obtained by dividing the residual activity by the initial activity represents the excess iron which has not reacted with the serum protein. The total iron-binding capacity (TIBC) is then determined from the following relation, previously described.

$$TIBC = \left(1 - \frac{S \times t}{T}\right) \times \mu\text{g. Fe added} \times 2 \times a = \mu\text{g. Fe/ml.}$$

The steps of Example II were followed to determine the total iron-binding capacity of sera withdrawn from a number of subjects. The following table presents the initial and residual activity as counts per minute, the TIBC and adsorbent correction factor, and the total iron-binding capacity of the respective sera.

	Initial activity, c.p.m.	Residual activity, c.p.m.	Correction factors		
			TIBC	Adsorbent	TIBC, g./ml.
Subject:					
1.....	27,410	16,041	1.17	0.9	3.62
2.....	27,530	15,959	1.17	0.9	3.69
3.....	28,037	16,548	1.17	0.9	3.55

The total amount of ferric ion added in each case was 6.37 μg.

A normal total iron-binding capacity is in the range 3.5 μg./ml. The total iron-binding capacity for sera representing various pathologic states is as follows: dietary iron-deficiency anemia: 4.50-5.00 μg./ml.; anemia of chronic infection: 1.50-2.00 μg./ml.; hemolytic anemias such as sickle cell anemia: 2.53 μg./ml.; thalassemia: 2.03 μg./ml.; and primary hemochromatosis: 2.00 μg./ml. Accordingly, the method of this invention can be used in the diagnosis and treatment of these and other disorders.

What is claimed is:

1. A method for determining the total iron-binding capacity of serum which comprises the steps of: adjusting the pH of a specimen of serum to the range of about 3.5±0.5 to release the bound iron; adding calcium carbonate adsorbent to the serum to bind the released iron and prevent the iron from recombining with the serum; adjusting the pH of the serum to the range of 7-8; separating the adsorbent and released iron from the serum; mixing a tracer amount of radioactive iron with the serum from which the iron has been removed; placing in intimate contact with said mixture an ion-exchange resin to remove the radioactive iron not bound by the serum proteins; incubating the resin-radioactive iron-serum mixture; measuring the initial radioactivity of the combined mixture with suitable detecting means; separating the ion-exchange resin from the mixture; washing the ion-exchange resin; and measuring the residual radioactivity in the ion-exchange resin.

2. The method of claim 1 wherein the resin-radioactive iron-serum mixture is incubated for about one hour at a temperature of about 45° C.

References Cited

- Nuclear Science Abstracts, vol. 23, July 1969 Item No. 26,658, abstracted from Brozovich, J. Clin. Pathol., 21, pp. 183-8 (March 1968).

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