CHAPTER 13

DIFFERENT METHODS FOR SERUM IRON ESTIMATION

Various methods have been adopted in past by different workers [2, 11, 19, 30, 43, 48, 54, 58, 72, 74, 78, 79, 81, 89, 96, 97, 99, 100, 106, 107] for the microestimation of iron in the serum. These methods usually vary in details from each other in one way or the other, nevertheless, the basic principle remains unchanged. Most of them are colorimetric methods and the reagents employed are ortho-phenanthroline, bathophenanthroline, 4:7 Diphenyl 1:10 phenanthroline, thiocyanate, thioglycollic acid or 2:2' dipyridyl to develop the colour. It is usual to obtain an optical density of 0.03 to 0.04 on analysing a sample containing 100 u g iron per 100 ml. (Trinder p. 1955).

IRON REAGENTS

(1) Ortho-phenanthroline :

Ortho-phenanthroline is ussd for serum iron estimation in Method of Davies et al.[43] The serum iron is liberated with hydrochloric acid and the protein-free filtrate is obtained after the precipitation of proteins. Then the ortho-phenathroline is allowed to react with ferrous iron in the serum in presence of sodium hydrosulphite, thus producing a red colour, the intensity of which is determined in the colorimeter. Gupta et al.[54] in their modified method of Hamilton et al.[58] used ortho-phenanthroline along with thioglycolic acid for the production of colour, and measured the optical density in Beckman Spectrophotometer at 510 m u.

(2) Bathophenanthroline :

This reagent was used in the method of Barkan and Walker.[11] The iron present in serum or plasma is liberated by 0.2 N hydrochloric acid and a drop of thioglycollic acid at room temperature. Then the proteins are precipitated with Trichloracetic acid. The iron present in the protein-free supernatent is determined by the addition of sodium acetate and alcoholic solution of Batho-phenanthroline, which is a reagent twice as sensitive for iron as compounds in general use.

(3) 4:7 Diphenyl 1:10 Phenanthroline :

4:7 Diphenyl 1:10 Phenanthroline is used in the method

of Peterson.[96] This reagent when allowed to react with the iron present in serum, gives a red colour,

(4) Thiocyanate :

Thiocynate was used by Powell [100] in his modification of method of Heilmeyer (1937). In this method the ferrous iron in the protein-free filtrate is converted to the ferric state, the ferric iron being determined by reaction with thiocynate.

(5) 2:2' Dipyridyl :

2:2' Dipyridyl is used by in the method described by Marrack.[78] In this method the ferrous iron is allowed to react with 2:2' dipyridyl. A pink colour is produced as a result of the reaction between the iron and the iron-reagent. The intensity of the pink colour so produced is compared with that produced by suitable iron standards.

DIFFERENT METHODS

(1) Heilmeyer's Method :

This method was described by Heilmeyer in 1937. In Heilmeyer's original method, the iron in the protein-free filtrate is reduced to the ferrous state, and determined colorimetrically with ortho-phenanthroline. The results

thus obtained have been criticized by Vahlquist (1941), on the grounds that, under Heilmeyer's conditions, complete reduction is not effected.[100]

(2) Barken and Walker's Method :

This method was described by Barken and Walker[11] in 1940. The iron from serum is liberated by the action of hydrochloric acid and a drop of thioglycollic acid. Proteins are precipitated by trichloracetic acid. Then iron is allowed to react with bathophenanthroline in presence of sodium acetate.

(3) Modification of Heilmeyer's Method :

Modified Heilmeyer's method was adopted by Powell[100] in 1944. Here, the ferrous iron present in the protein-free filtrate is converted to ferric state. This ferric iron is then allowed to react with thiocyanate. The properties of the ferric thiocyanate colour have been thoroughly studied by Moore, Minnich and Welch (1939). Under controlled conditions the colour intensity is proportional to the concentration, and is stable for one hour in the dark. It is also apparently unaffected by concentrations of phosphate upto five times those normally present in serum.[100]

(4) Method of Davies et al. :

This method for micro-estimation of serum iron was described by Davies, Levin and Oberholzer[43] in 1952. The

serum iron is estimated by liberation with HCl, and after removal of protein, ferrous iron is allowed to react with ortho-phenenthroline in presence of sodium hydrosulphite. The optical density is measured on SPEKKER absorptiometer.

(5) Peterson's Method :

In this method described by Peterson [96] in 1953, the then newly introduced reagent namely 4:7 diphenyl 1:10 phenanthroline was used for the determination of iron in serum. This reagent gives a red colour with ferrous iron having an optical density 2to 3 times as great as that obtained using conventional reagents, and the iron complex is insoluable in water and can be extracted and concentrated from an aqueous medium by means of amyl alcohol or hexanol. Some of the reagents used in the determination can be rendered iron-free by preliminary extraction with this colour reagent. These advantages are offset by the medium of extraction which introduces an extra stage in an analysis which is already sufficiently complicated (Trinder p. 1955).

In Peterson's method, the iron is extracted by precipitating the protein with hot trichloracetic acid - thioglycollic acid. After centrifuging and decanting the supernatant fluid, the protein precipitated is washed with a further portion of trichloracetic acid. Petersone stated that the percentage recovery of added iron decreases as increasing amounts of serum are precipitated from the same

final volume of solution.

(6) Trinder's Method :

Trinder introduced this method in 1955. He had adopted Peterson's method of protein precipitation but thioglycollic acid was not added to the trichloracetic acid, as it does not affect the recovery of iron and it causes some break-down of haem iron during the analysis. After adding trichloracetic acid, heating was carried out in a water bath at 90-95° c for 10 minutes. In this method also 4 : 7 diphenyl 1:10 phenanthroline was used as the iron reagent.

(7) Modified Method of Davies et al. :

This method was adopted by Mody et al.[89] in 1956. In the original method 0.2 ml. of serum was taken and the final volume for colorimetric estimation was 0.55 ml. In the modified method, the volumes of sera, reagents, solutions etc. were doubled and the final volume was made up to 1.5 ml.with glass-distilled water. Readings were taken on G.P.Photoelectric colorimeter using Ilford 404 green filter and microcuvette provided with an adapter. Instead of washing the glassware with 6N HCl, they were washed with chromic acid solution and finally rinsed thrice with glass-distilled water. In agreement with previous investigators, this method of washing was found quite suitable for the purpose.

(8) Modified Method of Heilmeyer and Plotner :

Johri [72] carried out the serum iron estimation in 1959 by the method of Heilmeyer and Plotner (1937) as modified by Laurell (1947). In this method, the phenanthroline reacts with the ferrous iron of serum in presence of sodium hydrosulphite, thus producing a red colour, the intensity of which is determined in the colorimeter.

IRON EXTRACTION PROCESSES

It is generally agreed that iron is present in serum in the form of iron-protein complexes, being non-dialysable, and only a small fraction appearing in the protein-free filtrate after treatment with trichloracetic acid. Vannotti and Delachaux (1942) have measured this fraction in a number of normal and pathological conditions and have suggested that it represents iron present in an active, lossely bound form. [100]

Determinations of total iron have been made on whole serum and plasma after ashing (Moore, Arrowsmith, Quilligan, and Read, 1937), but these methods are rather long and frequently involve a separate determination of haemoglobin iron, since it is not always possible to avoid haemolysis. Alternative methods entail preliminary treatment of the serum with mineral acid to detach the iron from its complexes,

followed by protein precipitation and analysis of the protein-free filtrate (Heilmeyer and Plotner, 1937; Barkan 1937; Vahlquist, 1941).

Most of the published methods for serum iron determination utilize first some technique for releasing the iron from its combination with the iron binding globulin, followed by precipitation of serum proteins. Strong (2 to 6 N) HCl has been used particularly by German and Scandinavian workers.[97]

This technique has been shown in study by Theodore Peters et al. (1956) and also by Bothwell and Mallett[19] to give complete extraction and to give results agreeing with other methods. However, the large amount of alkali required to neutralize the strong acid causes an appreciable increase in both the final volume and in the blank reading, factors both of which decrease the sensitivity.[97]

Use of dilute (0.2 to 0.3 N) HCl, introduced originally by Barkan,[9] is a satisfactory and simple procedure, but it gives low values when applied to stored serum specimens. The extraction procedure of the method proposed by Theodore Peters et al. (Loc. Cit.) is the same as that of Barkan and Walker,[11] except for the introduction of a reducing agent and use of room temperature instead of 38°C. It may be conjectured that fresh serum releases its iron at pH 1 to 2 more completely than does stored serum because of an oxidative process occurring incident to storing. Incubation at 38° C was not found to be necessary when a reducing agent was added.

Cartwright et. al.[97] reported recoveries of 95 to 103 per cent of added iron with the Barkan and Walker method, but Peterson[96] obtained values of only 83 to 93 per cent of Fe.[59] He did not state whether the sera were fresh or stored.

Reducing agents have been used previously to release the iron from its combination. Ramsay[107, 108] used sodium sulphite, and his method apparently gives reasonable values. It showed iron recoveries of only 95 per cent or 83 to 110 per cent,[107] however, and involves the inconveniences of preparing solutions daily and of boiling to precipitate the serum proteins. Peterson[96] adds thioglycolic acid along with trichloracetic acid, a procedure introduced by Thompsett in 1934. He makes use of the greater colour intensity of bathophenanthroline, but the procedure required heating and extraction of the colored complex with alcohol. He reported 85 to 98 per cent recoveries of added iron.

The method of kitzes, Elvehjem, and Schuette[79] employs boiling to denature the serum proteins and to release the iron prior to addition of trichloraacetic acid. It gave values lower than the proposed method[97] and variable iron recoveries averaging under 90 per cent. Other workers reported 64 to 92 per cent recovery of added iron or radioiron,[96] and values 30 to 60 mcg. per 100 ml. lower than those obtained with Ramsay's method.[107] Schade et al.[116] have published a procedure in which iron is released by means of ascorbic acid at pH 6, and

then the colour is developed with terpyridine without protein precipitation and read against a serum blank. It appears very simple in application, but would seem to suffer in sensitivity when the serum specimens are turbid or otherwise optically dense.[97]

Determination of iron by digestion or extraction with acid and color development with thiocyanate has been applied[30,74] but the iron-thiocyanate color is of low intensity and requires special measures such as alcoholic extraction of the complex.

METHOD OF MARRACK(1956)

The method for serum iron estimation described by Marrack (1956) deserves a complete discription as it is adopted, of course, after MODIFICATION, in the present study. The original method[78] is given below.

(1) Principle :

The iron is maintained in the reduced state with ascorbic acid. The pink colour of ferrous iron when treated with 2:2' dipyridyl is compared with that produced by suitable iron standards.

(2) Procedure :

Test : Take 2 c.c. serum in a small centrifuge tube and add 0.1 c.c. ascorbic acid solution. Mix carefully and allow to stand for five minutes. Add 0. 2 c.c. of trichloracetic acid solution. Stirr nicely at least for 45 seconds with a

thin glass-rod - allow to stand for 10 minutes, before centrifuging for a period of 20 minutes. 0.2 c.c. of alkaline acetate solution and 3 drops of dipyridyl reagent are placed in small test tube. 1 c.c. of supernatant fluid is added and the mixed contents are allowed to stand for 10 minutes before reading in a spectrophotometer at 520 m u, using the blank solution to set the instrument to zero.

Blank : 2 c.c. distilled water treated as serum, i.e. with 0.1 c.c. ascorbic acid and 0.2 c.c. trichloracetic acid, and 1 c.c. of mixture treated with 0.2 c.c. of acetate and 3 drops of dipyridyl.

Standards : 2 c.c. portions of solutions containing 100 and 200 ug. of iron per 100 c.c. treated as serum and read against the blank.

Calculation :

$$\text{Serum iron (ug. per 100 ml.)} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 100 \text{ (low standard)}$$

$$\text{OR} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 200 \text{ (high Std.)}$$

Apparatus and Solutions : Since the level of iron in serum is so low, contamination is the most important cause of inaccuracy. Keep a special set of syringes and glassware for this estimation. The apparatus should be boiled in dilute HCl, washed with distilled water, dried and protected from

dust. It may also be necessary to discard certain batches of reagents if they produce a strong colour in the blank.

Trichloracetic acid : 60 per cent (W/V) trichloracetic acid in water.

Alkaline Acetate Solution : N-NaOH is saturated with solid sodium acetate (25 g.per 10C c.c.). The solution is kept in a tall bottle and portions removed for use without disturbing the deposit which forms.

Ascorbic acid solution : A 'knife-point' of ascorbic acid (0.25 g) is dissolved in 3 c.c. of N-HCl. Solution is made freshly each day.

Dipyridyl Reagent : A small 'knife-point' of ascorbic acid (0.1 g) and 10-15 crystals (0.01 g) of Anala-R 2:2' dipyridyl are dissolved in 2 c.c. of distilled water. Solution is made freshly each day.

Iron standards : The 'dilute iron standard' described below contains 500 ug. Fe per ml. Standards containing 100 and 200 ug. per 100 c.c. are prepared by diluting 2 and 4 c.c. of this solution to 1 litre with 0. 01 N-HCl.

Iron Standard : The stock iron standard is an acid solution of Ferric ammonium sulphate (43.18 gm. dissolved in water with about 100 c.c. concentrated HCl and made to 1 litre), containing 5 mg. Fe per c. c. The dilute iron

standard is a 1 in 10 dilution of this.

REMARKS

Thus, it is clear from the foregoing discreption that various methods for serum iron estimation are quite complicated. The method described by Marrick (1956) involves the difficult procedure of boiling the glassware in 2.5 N HCl. Moreover, Marrack has neither given any details about the procedure for rendering the glassware iron-free nor has he described the procedure for standardisation. So is true for all the other methods described by various authors. These and many other important wants have therefore been fully satisfied in the present study (Section III, Chapter 14 to 19).