The Determination of Iron in Blood Plasma or Serum

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Some time ago it became necessary in this department to have a rapid and reliable method for the determination of iron in blood serum or plasma. The method was required for teaching purposes on human subjects and for investigations on iron metabolism in various species. Since none of the published techniques seemed quite suitable, a new one was based on the experience of the author (Ramsay, 1951, 1952) in determining non-haem iron in the chick embryo and its liver. The new method is described here, with illustrative examples of its use and comparisons with the results of others.

The separation of iron from the proteins of blood plasma has long been known to be a matter of difficulty. Barkan (1927) observed the serum iron to be neither dialysable nor ultrafiltrable, but claimed that it could be made so by incubation of the serum with dilute hydrochloric acid. Fowweather (1934) demonstrated the retention of a substantial proportion of the plasma iron by a trichloroacetic acid protein precipitate, and determined iron in the serum after wet ashing with sulphuric acid and hydrogen peroxide. This procedure is tedious, involves the use of relatively large quantities of reagents liable to be contaminated with iron, and may under certain conditions lead to loss of iron. A similar technique was employed by Moore, Arrowsmith, Quilligan & Read (1937). Tompsett (1934) believed that Fe^{3+}, but not Fe^{2+}, is adsorbed to the precipitated protein, and added thioglycollic acid before a trichloroacetic acid precipitation. The majority of later workers (e.g. Moore, Minnich & Welch, 1939; Barkan & Walker, 1940; Vahlquist, 1941; Powell, 1944; Laurell, 1947; Tompsett & McAllister, 1949) have made use of Barkan's (1927) observation and have incubated the plasma or serum for periods of 30 min. to 16 hr. with hydrochloric acid in concentrations ranging from less than 0·1N to about 2N before precipitating proteins with trichloroacetic acid. The possibility of subsequent physical or chemical reaction between the dissolved iron and the precipitated protein does not seem to have been envisaged, unless perhaps by Kitzes, Elvehjem & Schuette (1944), who heated the plasma to denature the proteins and extracted iron by two treatments with hot trichloroacetic acid. In all methods the iron is finally determined colorimetrically, with such reagents as *o*-phenanthroline, 2:2′-dipyridyl or thiocyanate. Table 1 gives some typical examples, chosen from the numbers scattered through the literature, of the results obtained by applying these methods to normal human subjects. It will be seen that the means range from 94 to 143 μg./100 ml., and that usually the values for women are lower than those for men. Results obtained by the use of the method described here suggest strongly that the true normal average is distinctly higher than the figures in Table 1.

The new method is based on the idea of replacing the metal-combining globulin of the plasma

Table 1. *Normal serum or plasma iron concentrations previously reported*

Authors	Method*	No. and sex of cases	Serum or plasma Fe (μg./100 ml.)	S.D.
Moore *et al.* (1937)	—	15 M.	122	26
		15 F.	98	24
Vahlquist (1941)	—	50 M.	142	43
		50 F.	120	32
Powell (1944)	—	35 M.	143	24
		35 F.	117	27
Cartwright, Huguley, Ashenbrucker, Fay & Wintrobe (1948)	Kitzes *et al.* (1944) Barkan & Walker (1940)	49 M. 43 F.	105 104	30 36
Cartwright & Wintrobe (1949)	Barkan & Walker (1940)	15 M.	127	29
		15 F	123	19
Rath & Finch (1949)	Kitzes *et al.* (1944)	15 M.	106	18
		15 F.	94	19

* No reference is given in this column when the authors quoted used their own method.

(Surgenor, Koechlin & Strong, 1949) by another co-ordinating reagent under conditions which render reversibility of the reaction very unlikely. 2:2′-Dipyridyl (first used for iron determinations by Hill, 1931) was selected because it reacts only with ferrous iron, and excess reducing agent must be added which will prevent any reversion to the original ferric form; because the ferrous-dipyridyl complex ion is highly coloured; and because the complex is quite stable at a pH which permits the removal of the plasma proteins by filtration after coagulation by heat.

METHOD

It must be emphasized that, if interference from extraneous iron is to be avoided, the following precautions are obligatory: all glassware must be cleaned in the usual way, boiled with 5N-HCl (A.R.), and rinsed with distilled water only. HNO_3 may be used instead of HCl, but cleaning by soaking in $K_2Cr_2O_7.H_2SO_4$ is not an adequate substitute for the final acid treatment. All chemicals used should be of A.R. grade, and must be tested to ensure their comparative freedom from iron. It is important to use an aluminium or other non-ferrous water bath, and to fill this with distilled water. The reagent 'blank' in this laboratory amounts to about 0·3 μg./estimation.

The method is suitable for application to serum, or to plasma from heparinized or oxalated blood. Haemolysed specimens should not be used, as some of the haem Fe present may react under the usual conditions. In sera of no more than average bilirubin content, as little as 20 μg. oxyhaemoglobin Fe/100 ml. may be detected by eye, but in cases of doubt a pocket spectroscope should be used.

Reagent. Acetate buffer 0·5M, pH 5, containing 2:2′-dipyridyl, 0·075%, and hydroxylamine HCl, 0·1%. The composition of the reagent is not critical in any respect. It is stable for months in a brown bottle, kept away from direct sunlight.

Sufficiently accurate standard solutions may be made from either ferrous or ferric ammonium sulphate.

Procedure. Plasma or serum (2 ml.) is pipetted into a tube (5 × ½ in.) graduated at 7·5 ml. The reagent (5 ml.) is added, and water to the mark. The contents are thoroughly mixed by careful stirring, and the tube is heated for 5 min. in a boiling-water bath in such a way that the water level is above the graduation on the tube. During the heating the ferrous dipyridyl complex is formed and the proteins are coagulated. The tube is allowed to cool thoroughly, and sharply shaken to break up the gelatinous precipitate, which is then removed by filtration through a 7 cm. paper (no. 42; coarser papers, and those not washed with HCl, are unsuitable). The intensity of the pink colour is measured in the Unicam D.G. spectrophotometer set to 520 mμ., using 1 cm. cells. For other photometric apparatus it may be necessary to scale up the method, as these cells conveniently require only 3 ml. fluid. For instruments which employ filters, Ilford 604 or 624 should be chosen. For a 'blank' solution, an appropriate mixture of the reagent and distilled water is used. If minimal quantities of sodium oxalate (A.R.) or heparin ('Liquemin' Roche) are used as anticoagulants, these introduce no error and need not be included in the 'blank'.

RESULTS

Reproducibility. The standard deviation of a single determination from the mean, calculated indirectly as suggested by Dahlberg (1940) from seventy consecutive duplicate analyses on specimens of human plasma from oxalated blood, was ± 5·1 μg./100 ml. blood, or about 2·8%. Some routine duplicate analyses may be seen in Tables 3, 5 and 6. Twelve analyses on a single specimen of sheep serum (which contains more iron than human serum) gave the following results: 320, 310, 300, 323, 304, 300, 313, 300, 317, 317, 304 and 362 μg./100 ml. All these results lie close to the mean except the last, which is about six standard deviations (S.D. ± 8·6, 2·8%) higher than the mean (310 μg./100 ml.) obtained if it is omitted from the calculations. Such discrepancies are very rare.

Recoveries. Table 2 gives the results of a number of recovery experiments in which small volumes (0·1–0·3 ml.) of standard ferric ammonium sulphate solution were added to 2 ml. portions of plasma. The quantities of iron added were kept small enough to be well within the iron-binding capacities of the plasmas analysed. The total concentration of iron, including the added material, ranged from 205 to 265 μg./100 ml. It was felt that recoveries of larger amounts of iron would not form a sound test of the method, and that under these difficult circumstances recoveries of 83–110% may be considered very satisfactory.

Table 2. *Recoveries of iron added to 2 ml. plasma*

Plasma	Fe added (μg.)	Fe recovered (μg.)	%
A	1·66	1·38	83
	1·66	1·49	90
B	1·66	1·83	110
	1·66	1·58	95
C	2·00	1·94	97
D	2·00	1·99	99·5
	2·00	1·99	99·5
E	2·66	2·77	104
	2·90	2·90	100
F	3·33	3·55	107
	3·33	3·30	99

Comparison of serum and plasma. In Table 3 are presented figures which show that serum, oxalate plasma and heparin plasma give virtually identical results. For these analyses a single specimen of blood was drawn into a 20 ml. syringe and divided into separate portions afterwards. The figures also show that oxalate and heparin in the low concentrations used for the inhibition of the coagulation of blood neither interfere with the determination nor contribute significant quantities of iron to the 'blank'.

Table 3. *Iron content (μg. Fe/100 ml.) of serum, oxalate plasma and heparin plasma*

Serum	Oxalate plasma	Heparin plasma
216	—	207
191	—	187
—	139	139
—	142, 145	145, 152
—	145, 145	149, 149
—	200, 194	207, 203
113	110	—
174, 181	178, 184	—
203, 207	200	—
129	129, 126	—

Effect of haemolysis. A number of experiments were done in which measured quantities of haemolysed erythrocytes (standardized for total iron by the method of Ramsay, 1952) were added to plasma or serum of known iron concentration. The mixtures were analysed for plasma iron and observed visually and spectroscopically. It is clear from the results of the experiment quoted in Table 4 that no danger need be expected from haemolysis unless this is easily perceptible.

were selected. The first is typical of the hydrochloric acid-incubation techniques, and although the second is based on rather different principles the two have been found (see Table 1) to give results of the same order. It is obvious from Table 5 that the new method does in fact give higher results, although the values obtained by the use of the published methods are similar to those now accepted. Oxalate plasma was used for the comparisons with the method of Kitzes *et al.* (1944), serum for those with that of Barkan & Walker (1940).

Diurnal variation. It has frequently been claimed that the plasma iron varies daily in that early morning values are higher than those obtained in the afternoon or evening. The most extensive investigations of this point have been made by Hoyer (1944), and the most recent by Hamilton, Gubler, Cartwright & Wintrobe (1950). The matter has been the subject of controversy, and seemed suitable for investigation by the new method. Blood specimens taken from each of seven normal male subjects at 8.45 a.m. after a light breakfast and again at 5.15 p.m. were analysed.

Table 4. *Effect of haemolysis on plasma iron*

Haem Fe added (μg./100 ml.)	Appearance of plasma	Spectroscopic examination	Plasma Fe (μg./100 ml.)
0	Deep yellow; turbid	—	258, 255, 258
15	Orange-yellow	α-Band of HbO_2 doubtful	258, 265
37·5	Deeper orange-yellow	α-Band visible; β-band doubtful	268, 265
75	Orange	Both bands clearly visible	281
112·5	Pinkish orange	Both bands marked	304, 314

Comparison with other methods. As the general level of the results obtained by the new method was distinctly higher than the accepted averages, it

Table 5. *Comparison with earlier methods*

Serum or plasma Fe (μg./100 ml.)		
Barkan & Walker (1940) (serum)	Kitzes *et al.* (1944) (plasma)	This method
52, 57	—	110, 113
106, 111	—	142, 148
107	—	191
165, 176	—	229, 236
157, 159	—	210, 203
159, 147	—	203, 207
—	109, 122	178, 168
—	72	124
—	111	156
—	183	216
—	126, 108	181, 174
—	90, 84	178, 171
—	112	184
—	136, 136	174, 171

seemed important to make comparisons on the same specimens of plasma. For this purpose the methods of Barkan & Walker (1940) and Kitzes *et al.* (1944)

The experiments were done at different times and run in conjunction with other analyses, none of which showed any unexpected trend. In all seven cases the afternoon plasma specimen contained less

Table 6. *Diurnal variation in plasma iron concentrations*

Subject	Plasma Fe (μg./100 ml.) 8.45 a.m.	5.15 p.m.
G.B.	150, 156	131, 131
W.R.	—, 122	106, 106
G.W.	137, 134	99, 100
A.P.	156, 153	143, 137
D.P.	255, 249	159, 162
R.I.	201, 190	153, 146
A.R.	142, 136	116, 123

iron than the morning one. It is true that in one case the decrease (9 %) was small, but in the other six it ranged from 14 to 37 %, with a mean of 19 %. There can be no doubt that the diurnal variation is real.

Normal values. Males. The twenty-two specimens analysed were from seventeen normal males between the ages of 19 and 44 years. Because of the

diurnal variation, all specimens were taken between 8.45 and 9.15 a.m. Haemoglobin concentrations (calculated from total blood iron) ranged from 14 to 17 g./100 ml. blood. The mean plasma iron concentration was 171 μg./100 ml. (S.D. 37), and the values ranged from 112 to 255 μg./100 ml. An isolated value of 346 μg./100 ml. (about 4·5 standard deviations higher than the mean) was not included in the series, although such very high values have occasionally been recorded before. Hamilton *et al.* (1950) observed one, also in the early morning, of 355 μg./100 ml.

Females. The three specimens analysed were taken from thirteen apparently normal females aged between 19 and 24 years under the same conditions. Haemoglobin concentrations ranged from 12·2 to 14·9 g./100 ml. The plasma iron concentrations ranged from 78 to 170 μg./100 ml., with a mean of 127 μg./100 ml. (S.D. 29 μg./100 ml.). As in all previous work, these values are lower than those for males.

Iron absorption. The method lends itself readily to the demonstration that iron administered by mouth as colloidal ferric hydroxide is more readily absorbed if ascorbic acid is fed at the same time. Experiments on this point were made by analysing blood specimens before, and at intervals up to 7 hr. after, a dose of 200 mg. iron as colloidal ferric hydroxide taken with 600 mg. ascorbic acid. Control experiments were done without ascorbic acid, and the results plotted graphically, as in Fig. 1. In the absence of ascorbic acid the plasma iron rose by 20–50 μg./100 ml. in 3–5 hr., and returned to the original level in about 7 hr. When ascorbic acid was given with the iron, the increase in plasma iron was much greater (some 100–200 μg./100 ml.), and the normal level was never regained in 7 hr. The fall in the latter part of the experiment, however, was precipitate, so that it seemed likely that the normal level would be reached in 8–9 hr.

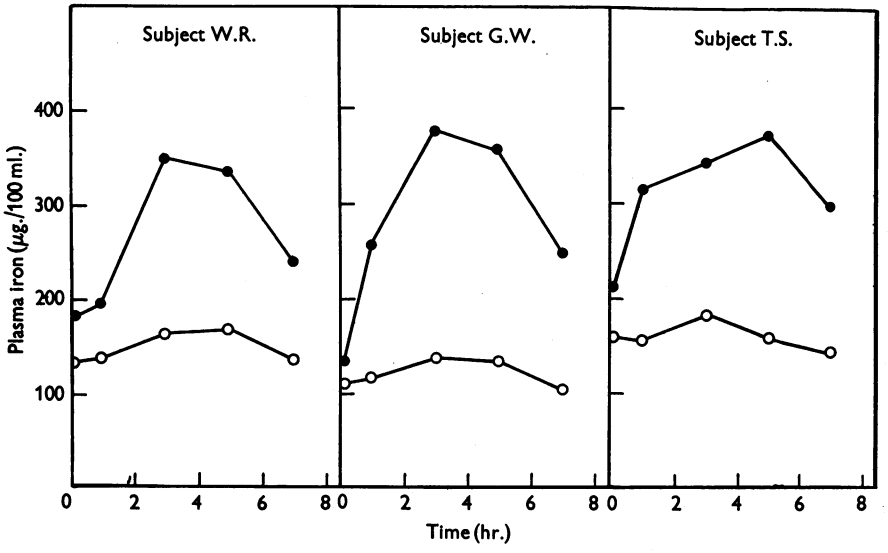

Fig. 1. Plasma iron curves after feeding 200 mg. Fe as colloidal ferric hydroxide with (●), and without (○), 600 mg. ascorbic acid.

DISCUSSION

The technique described here is simple and gives highly reproducible results, provided that strict attention is paid to the cleansing of glassware. Recoveries of added iron are satisfactory, and interest must centre chiefly round the fact that it gives results higher by some 30–60 μg./100 ml. than those previously published. The author believes this difference to be real and important. The figures in Table 4 show that the degree of haemolysis necessary to cause a spurious elevation of this order would be quite evident to the naked eye. Moreover, if haemolysis or contamination with totally extraneous iron were responsible for the general high level of results, one would have expected occasional quite substantial inconsistencies in experiments involving serial determinations. Such inconsistencies have not been encountered. Finally, experimental proof has been obtained that the extraction procedure of Kitzes *et al.* (1944) does not remove all the iron from the protein precipitate. Thus, in one case the plasma iron according to Kitzes *et al.* (1944) was 183 μg./100 ml. Two additional extractions with hot trichloroacetic acid brought the value up to 222 μg./100 ml., while the

new method gave 216 μg./100 ml. The fact that haemolysis is a real hazard in this otherwise useful procedure is nevertheless a cause for disquiet, and the author is at present engaged in the development of a modification which may, for all practical purposes, eliminate this difficulty.

The diurnal variation has been observed by a number of previous authors, whose work was reviewed by Hamilton *et al.* (1950). Failure to detect it by Moore *et al.* (1939) and Burch, Lowry, Bessey & Berson (1949) can only be attributed to inadequate analytical technique. Its existence makes it imperative to standardize the time of day at which blood specimens are drawn for the determination of plasma iron. There is no evidence that the majority of authors have in the past paid due attention to this point, which has also been raised by Hoyer (1944) and by Hamilton *et al.* (1950). It is necessary to state, however, that there are other, unknown, influences which tend to make the plasma iron concentration remarkably variable. In the course of the present work specimens from certain individual males have been examined more than a dozen times at irregular intervals over a period of 2 years. In one such case the values obtained have varied from 112 to 180 μg./100 ml., while in another the variation has been from 122 to 235 μg./100 ml. Large variations have also been encountered by other workers (Hoyer, 1944).

The effect of ascorbic acid on the absorption of iron administered as ferric hydroxide is striking. It is interpreted as a manifestation of the reducing action of ascorbic acid, which, according to Bergeim & Kirch (1949) is likely, under natural conditions, to supplement to a marked extent the reducing agents formed from proteins by normal gastric juice. While it seems probable that the chief effect of ascorbic acid will be on the reduction of Fe^{3+} to Fe^{2+} within the lumen of the intestine, the possibility should not be ignored that there may also be an effect on 'the so-called redox level of the cell' (Granick, 1949, 1951). There is no experimental evidence which would permit a decision on the relative importance of these ideas.

SUMMARY

1. A new, simple method for the determination of iron in serum or plasma is described.

2. Normal values and comparisons with earlier methods show the results to be higher than those now generally accepted.

3. The method is readily applied to studies involving the determination of serum or plasma iron.

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