Spectrophotometric Determination of Iron in Serum

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For the determination of iron in serum, various methods have been proposed. They cover photometry or colorimetry applied to the colored solution obtained by adding a color-developing reagent, such as thiocyanate[1-4], *o*-phenanthroline[5-11], α,α'-dipyridyl[12,13] or 4,7-dimethyl-1,10-phenanthroline[14] to the serum after the removal of protein. In ordinary cases the iron content of serum is low and the available amount of serum is small. When the methods mentioned above are applied to a few ml. of serum, the color of the solution is very feeble. Therefore, it is difficult to obtain precise results unless a very sensitive photometer or colorimeter is available. Hence, a method was devised by Jones[2], which is based on light absorption of colored extract obtained by the extraction of iron thiocyanate from the aqueous solution to the solvent immiscible with water. In this method, however, the decolorization of the solution occurs before the extraction is complete, because of the instability of iron thiocyanate in an aqueous medium.

Here the authors propose to apply to serum Torii's method for determination of iron[15,16] which was published recently. The method is based on the comparison of light absorptions of green solutions obtained by the reaction of *o*-nitroso resorcinmonomethylether (hereafter N. R. M. E.) with ferrous iron.

Reagents

Distilled water.—Redistilled water is obtained by using the water-distilling apparatus made of hard glass.

Standard iron solution.—In 10 ml. of sulfuric acid (1 : 10), 0.1015 gram of analytical-grade iron wire is dissolved for 20 hours' digestion. To the iron solution is added 3 ml. of concentrated nitric acid and the resulting solution is diluted to 500 ml..

6 N hydrochloric acid.—Distilled hydrochloric acid is titrated with alkali, and the hydrochloric acid of a known concentration is diluted to 6 N.

20% trichloroacetic acid.—Twenty grams of trichloroacetic acid is dissolved in 100 ml. of distilled water.

10% hydroxylamine hydrochloride.—Ten grams of hydroxylamine hydrochloride is dissolved in 100 ml. of distilled water.

0.1% p-nitrophenol.—One tenth gram of *p*-nitrophenol is dissolved in 100 ml. of distilled water.

6 N ammonium hydroxide.—Forty ml. of conc. ammonium hydroxide is diluted to 100 ml..

Acetic acid-sodium acetate buffer solution.—In 200 ml. of distilled water, 33.5 grams of sodium acetate is dissolved and the resulting solution is filtered. After addition of 27.2 ml. of distilled acetic acid, it is diluted to 250 ml..

5% sodium thiosulfate solution.—Five grams of sodium thiosulfate is dissolved in 100 ml. of distilled water and the resulting solution is filtered.

Saturated aqueous solution of N. R. M. E..—N. R. M. E. is dissolved in 100 ml. of distilled water andt he insoluble matter is filtered off (solubility: 40.2 mg./100 ml.).

Carbon tetrachloride.—Distilled carbon tetrachloride is obtained using a distilling apparatus made of hard glass.

Procedure and Apparatus

In a centrifuge tube, 2.0 ml. of clear serum is taken, acidified with 0.5 ml. of 6 N hydrochloric acid, and allowed to stand for 10 minutes. To the resulting solution is added 1.0 ml. of 20% trichloroacetic acid†. The mixture is centrifuged and filtered, if necessary. The precipitate is

† When serum is not acidified with hydrochloric acid and is treated only with trichloroacetic acid, the mixture is kept in a water bath for 5 minutes at 100°C.

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washed with a mixture of 1.0 ml. of distilled water and 0.5 ml. of 20% trichloroacetic acid. (Filteration and washing are not always necessary when the centrifugation is complete.) The clear filtrate, together with washing solution, is taken in a 10-ml. stoppered measuring cylinder. After addition of 0.5 ml. of 10% hydroxylamine hydrochloride, the solution is kept for at least 15 min. with shaking at certain intervals until the reduction of iron is complete. The mixture is then neutralized with 6 N ammonium hydroxide, the neutralization being indicated by the development of color of 0.1% *p*-nitrophenol (one drop) added to the solution.

For the pH control, 2.0 ml. of acetic acid-sodium acetate buffer solution (pH 4.5) is added. To this mixture are added 0.5 ml. of 5% sodium thiosulfate, and, after 5 minutes, 1.0 ml. of saturated aqueous solution of N. R. M. E.. The volume of the resulting solution was adjusted exactly to 10.0 ml. by adding distilled water. After being allowed to stand for at least 15 minutes†† the, mixture is transferred into a 30-ml. separatory funnel, and shaken with 2 ml. of carbon tetrachloride. Then the N. R. M. E. solution in carbon tetrachloride is discarded. The extraction is repeated three times to remove the excess of N. R. M. E. completely. The aqueous solution is poured into a centrifuge tube and is centrifuged to remove the suspended carbon tetrachloride. The supernatant aqueous solution is transferred to a 20-mm. absorption cell, and its absorption is measured at 700 mμ with Shimadzu's photoelectric spectrophotometer.

Examination

The following experiments were carried out with iron standard solution and serum at room temperature of 27–30°C.

a) To check the possibility of rapid reduction of ferric iron by sodium thiosulfate in the absence of hydroxylamine hydrochloride, the color was developed by the method mentioned above without adding hydroxylamine hydrochloride to iron standard solutions. The result seems to show that one hour is necessary for complete reduction of iron by sodium thiosulfate. (Table I)

TABLE I

ABSORBANCY OF SOLUTION OBTAINED BY THE REDUCTION OF IRON WITH BOTH HYDROXYLAMINE HYDROCHLORIDE AND SODIUM THIOSULFATE OR WITH SODIUM THIOSULFATE ALONE

Time in min.	15	30	60
Hydroxylamine hydrochloride and sodium thiosulfate	0.235	0.234	0.236
	0.184	0.185	0.184
Sodium thiosulfate	0.182	0.220	0.234*
	0.144	0.177	0.185**

* To be compared with 1st line.
** To be compared with 2nd line.

†† Not for as long a time as two or three days, which was the private suggestion given by T. Torii.

b) After the pH of solution is adjusted to 4.5, hydroxylamine hydrochloride was added to reduce iron in the standard solution. One hour was necessary for complete reduction. (Table II)

TABLE II

ABSORBANCY OF SOLUTION OBTAINED BY THE REDUCTION OF IRON IN STRONG ACIDIC MEDIUM AND pH 4.5

Time in min.	15	30	60
Strong acidic medium	0.208	0.210	0.209
	0.156	0.159	0.158
pH 4.5	0.149	0.183	0.210*
	0.116	0.139	0.158**

* To be compared with 1st line.
** To be compared with 2nd line.

From the results of experiments a) and b), it was found that iron was reduced completely within a short time by hydroxylamine hydrochloride in a strongly acidic medium as was used in the procedure mentioned above.

c) In the presence of 0.5 ml. of 6 N hydrochloric acid, the addition of 1.0 ml. of 20 % trichloroacetic acid to 2.0 ml. of serum is satisfactory to remove protein from serum. It was checked qualitatively by using sulfosalicylic acid. Under this condition, iron is liberated in solution completely by Surgenor et al.[17].

d) To check the effect of hydrochloric acid concentration on the development of color, each 2.0 ml. of serum was treated with 0.5 ml. or 1.0 ml. of 6 N hydrochloric acid. The subsequent treatment was the same as in the procedure mentioned above for each 2.0 ml. of serum. The results are shown in Table III, and no serious difference was found between these two conditions.

TABLE III

ABSORBANCY OF SOLUTION OBTAINED BY ADDING 0.5 ML. AND 1.0 ML. OF HYDROCHLORIC ACID TO SERUM

Serum	I	II	III	IV
0.5 ml. HCl	0.191	0.170	0.124	0.133
1.0 ml. HCl	0.186	0.168	0.126	0.136

e) In the determination of iron by Torii's method, the presence of copper in larger quantities than iron interfere with the determination. In human serum, the copper content is 1–1.5 times as great as that of iron. To see if addition of 5% sodium thiosulfate 0.5 ml. is sufficient for

17) D. M. Surgenor, B. A. Koechlin and L. E. Strong, *J. Clin. Investigation*, **28**, 73 (1949).

the prevention from interference of copper. The experimental results are shown in Table IV, and the corresponding absorbancies obtained for these two sets of solution are the same. Therefore it is clear that copper in human serum will not interfere seriously.

TABLE IV

ABSOREANCY OF SOLUTION OBTAINED FROM IRON STANDARD SOLUTIONS CONTAINING AND FREE FROM COPPER

Concentration of iron (γ/100 ml.)	81.2	121.8	162.3	203.0
Iron solution free from copper	0.110	0.160	0.204	0.258
	0.105	0.156	0.210	0.261
	0.100	0.152	0.217	0.264
Iron solution containing copper (300 γ/100 ml. Cu)	0.108	0.161	0.210	0.260
	0.110	0.160	0.214	0.266
	0.112	0.156	0.218	0.272

f) Based on the method proposed here, the standard solutions were used for the preparation of a calibration curve. The experimental results corrected for the blank test (absorbancy, 0.044) are shown in Fig. 1. The curve in Fig. 1 is linear and obeys Beer's law within the range of iron content from 0 to 800 γ per 100 ml.. The method presented here is advantageous over the usual one. The absorptions to be measured in the present method, are from two to three times as strong as those in the usual methods for a given content of iron in solution.

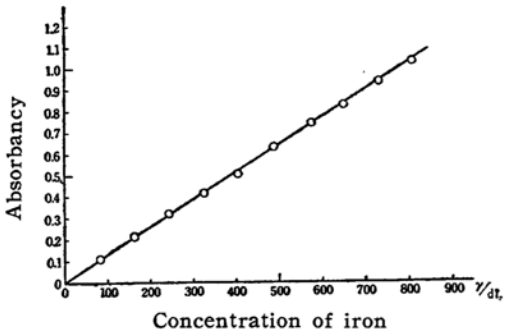

Fig. 1.

g) Recovery experiments were run in which iron was added to four samples of serum. In this set of analysis, a few ml. of a standard iron solution was added to a few ml. of the serum so as to made the total volume 2 ml. and this mixture was then analysed. The results in Table V show quantitative recovery.

TABLE V

RECOVERY OF KNOWN QUANTITY OF IRON ADDED TO SERUM

Serum volume ml.	Iron solution added. concentration γ/100 ml.	Iron solution added. volume ml.	Determined iron γ/100 ml.	Calculated iron from the value of pure serum γ/100 ml.
2.0			71	
1.0	406.0	1.0	235	238
0.6	406.0	1.4	299	305
0.4	406.0	1.6	345	339
2.0			135	
1.0	406.0	1.0	275	270
2.0			95	
1.0	406.0	1.0	128	129
2.0			102	
1.8	162.3	0.2	106	108
1.8	243.6	0.2	116	116
1.8	324.8	0.2	126	124
1.8	406.0	0.2	129	132

Summary

To improve the procedure of photometric determination of iron in human serum, Torii's method recently proposed for the determination of iron was applied to serum. After the removal of protein from serum, the iron in serum is reduced, the pH of the solution is adjusted to 4.5, the color-developing reagent is added, and the excess of the reagent is removed by extraction with carbon tetrachloride. The green solution thus obtained is effective for spectrophotometric determination.

The interference of copper is avoided by the addition of thiosulfate, and the substances other than copper remaining after the removal of protein from serum do not give any appreciable interference.

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