NEW METHOD FOR DETERMINING TOTAL IRON-BINDING CAPACITY OF SERUM (TIBC) WITH RADIOIRON BY ELIMINATING IRON FROM TRANSFERRIN

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The following in vitro methods have been used to determine the total iron-binding capacity of serum: (A) Excess iron is added to serum to saturate the iron-binding capacity. The unbound iron is removed by using phenanthroline (*1,2*) or iron-exchange resin (*3*) or magnesium carbonate (*4*), and then the bound iron is extracted and determined colorimetrically. (B) The amount of serum-iron (SI) (*2,5–9*) and the unsaturated iron-binding capacity of serum (UIBC) (*10–18*) are determined, respectively, and then the two values are added (*19*). (C) A method to measure transferrin quantitatively by using an antigen-antibody reaction has been devised to calculate TIBC (*19–21*). These methods for the routine determination of TIBC are relatively difficult to perform and are rather time consuming. This paper describes a simple method to remove serum-iron without the loss of iron-binding capacity of transferrin which makes possible direct and rapid determination of TIBC.

METHOD OF INVESTIGATION

Effect of desferrioxamine on removal of serum iron. Attempts were made to remove iron from transferrin at neutral pH by using desferrioxamine (DFO) (*22*), an iron-chelating agent. The DFO failed to remove iron from transferrin, and complete removal of DFO from serum was extremely difficult. Even if iron is removed by an iron-chelating agent without damaging transferrin, removal of the chelating agent will be difficult. These attempts were therefore abandoned.

Effect of various acids. Below pH 5.5 iron does not combine with transferrin. The iron-binding capacity of transferrin was not always recovered when it was treated in a too strongly acidic solution. Some reducing acids were conceived to be more effective than ordinary acids in terms of ability to release iron from transferrin. Reagents such as thioglycolic acid and hydroxylamine hydrochloride were tested, but their use resulted in a decrease of TIBC. A 2% ascorbic acid solution, with a pH of approximately 3, was effective. The fresh ascorbic acid solution was added to serum in equivolume. The pH of this mixture was approximately 5.5. For these studies a highly soluble ascorbic acid in granular form was used*.

Ammonium sulfate solution to remove iron was studied. Saturated ammonium sulfate solution was added to the acidified serum with ascorbic acid, and transferrin was precipitated. The supernatant was decanted, and the precipitate was dissolved in water. The percent of iron removal effectiveness using the two cycles of precipitation and dissolution was over 90%. This procedure, however, is too troublesome to carry out routinely. Calcium carbonate was also tested, but it failed to remove iron from the serum solution, acidified with ascorbic acid.

Amberlite IR-120 granules were evaluated for their ability to remove iron ion in acidified serum. IR-120 was soaked in *N* hydrochloric acid and washed repeatedly for days. However, when 0.2 gm of this washed IR-120 was added to serum, it became strongly acidic, and a decrease in TIBC was found although iron was removed. This phenomenon may be caused by the denaturation of transferrin. To avoid a decreased TIBC, resins were tested without soaking in hydrochloric acid. Because powder possesses a greater active surface area than granule, Amberlite CG-120 Type-3 was added to serum without any pretreatment.

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* "Hicee" manufactured by Takeda Pharmaceutical Co., Ltd., Osaka, Japan. One gram of "Hicee" contains 250 mg of ascorbic acid, 5 mg of calcium pantothenate, and 745 mg of excipient.

TABLE 1. EFFECT OF UIBC SATURATION ON TIBC VALUES

Conditions	μg/100 ml		
	Serum A	Serum B	Serum C
UIBC saturated with iron	234	392	273
Not saturated	230	385	273
Not saturated	234	393	283

For the percent removal of iron, transferrin was labeled with ^{59}Fe and the decrease of the radioactivity was used as an indicator. The removal of iron was 95% in 10–30 min at 21°C, and it was 96% in 10 min, 97% in 20 min, and 98% in 30 min at 37°C. From these experiments it was shown that Amberlite CG-120 Type-3 was effective in removing iron from transferrin without the loss of its iron-binding capacity. No significant decrease in the amount of transferrin was observed by immunoassay* in the serum after the treatment with 1.8% ascorbic acid and 2% sodium bicarbonate solution. The use of charcoal or florisil was not effective in removing iron.

TIBC determination. Radioactive ferric ammonium citrate† and sodium bicarbonate solution were added to iron-free serum to saturate TIBC. The unbound iron was removed by 0.25 gm each of wet Amberlite IRP-67 and CG-120 Type-3 powder‡ IRP-67 removed 96%, CG-120 Type-3 removed 74%, and the mixture of IRP-67 and CG-120 Type-3 powder removed more than 98% of 5-μg excess iron in iron-saturated serum solution at room temperature in 5 min. Magnesium carbonate was not effective as the mixture of IRP-67 and CG-120 Type-3 in the elimination of unbound iron.

The method described below for the determination of TIBC was established from results obtained in the preceding studies.

METHOD

Removal of serum iron. While the test tube is shaken, 2 ml of 1.8% fresh ascorbic acid solution are added to 2 ml of serum. (Do not add serum into ascorbic acid solution.) One-half gram dry CG-120 Type-3 powder is added to the acidified serum. Mix well. The mixture is incubated at 37°C for 15 min, stirred occasionally, and then the mixture is spun at 3,000 rpm for 5 min by a centrifuge with a radius of 12 cm or longer. Two milliliters of the supernatant solution is obtained.

Determination of TIBC. One milliliter of ^{59}Fe-ferric ammonium citrate containing 6 μg of iron is added to 2 ml of the above supernatant solution (equivalent to 1 ml of serum), and then 1 ml of 2% sodium bicarbonate solution is added. Mix well. Incubate at 37°C for over 15 min. One-fourth gram each of dry Amberlite CG-120 Type-3 and wet IRP-67 powder which have been thoroughly swollen with water and from which excess water has been removed by centrifugation in a filtration funnel is added to the mixture. The mixture is stirred with an automatic mixer*, let stand for 5 min, and centrifuged. After centrifugation for 5 min, 2 ml of the supernatant solution is transferred to a counting tube and then the radioactivity is counted in a well scintillation counter.

The TIBC is calculated as follows:

$$\text{TIBC} = \frac{\text{Fe content of std. Fe solution } (\mu g/100 \text{ ml}) \times \text{cpm in 2-ml supernant solution} \times 2}{\text{cpm in 2 ml of standard iron solution}}.$$

To make 2 ml of standard solution, 1 ml of water and 1 ml of ferric ammonium citrate solution are added. Counting the standard iron solution is necessary, but pre-counting of each sample is not required.

Experimental results and discussion. Using this method, the TIBC was determined. No significant differences were observed between the TIBC value

* Model TB-I, Taiyo-Bussan K.K., Kanda, Tokyo, Japan.

TABLE 2. COMPARISON OF DIRECT DETERMINATION OF TIBC VERSUS CALCULATION (SI + UIBC)

Hematologically normal female	TIBC (μg/100 ml)		SI (μg/100 ml). Colorimetric	UIBC (μg/100 ml).
	^{59}Fe method	SI + UIBC		^{59}Fe method
1	271	271	61	210
2	265	264	87	177
3	277	276	70	206
4	281	272	60	212
5	259	252	62	190
6	258	255	98	157
7	291	285	67	218
8	282	288	63	225
9	267	268	62	206
10	334	358	107	251
Average values	279 ± 27	279 ± 29	74 ± 16	205 ± 20

* Partigen-Transferrin, Behringwerke, A.G., Marburg-Lahn, Germany.

† Prepared in our laboratory using ^{59}Fe purchased at Oak Ridge, Tenn. (*16*).

‡ Rohm and Haas Co., Philadelphia, Penna.

TABLE 3. TIBC DETERMINATION IN VARIOUS DISEASE STATES

Diseases	TIBC (μg/100 ml) ^{59}Fe method	TIBC (μg/100 ml) SI + UIBC	SI (μg/100 ml) Colorimetric	UIBC (μg/100 ml) ^{59}Fe method
Hemochromatosis	244	244	220	24
Myelofibrosis	269	267	60	207
Acute myelocytic leukemia	216	216	70	146
Lymphosarcoma	221	215	30	185
Hemolytic anemia	364	364	41	323
Hypoplastic anemia	289	281	87	194
Hypoplastic anemia	248	242	148	94
Hypoplastic anemia	216	214	105	109
Iron deficiency anemia	309	309	21	288
Iron deficiency anemia	393	400	6	384
Iron deficiency anemia	310	306	12	294
Liver cirrhosis	230	237	160	77
Nephrosis	93	91	42	49

in the group in which UIBC was saturated with iron and that in the unsaturated group. Experimental results are shown in Table 1.

The TIBC was determined in normal persons and patients suffering from various diseases. No significant difference was observed between the TIBC value of the group determined by the present method and that of the group in which TIBC was obtained by adding SI to UIBC. SI was determined by the method of Matsubara (*9*), and UIBC was determined by the slight modification of Tauxe (*16*). The experimental results are shown in Tables 2 and 3.

To measure TIBC by colorimetric methods, many reagents are necessary and the procedures are entirely different from the routine UIBC method. Although the determination of TIBC by immune reaction can be performed using a small amount of serum, it takes 48 hr to obtain the results. Individual determination of both SI and UIBC is also troublesome to carry out. The present method is as simple as the routine UIBC method and can be determined directly after simply removing iron.

SUMMARY

A simple method for removing serum iron is described. To dissociate iron from transferrin, fresh ascorbic acid solution is used and ionized iron is removed with Amberlite CG-120 Type-3 powder. The iron-free serum solution was obtained by centrifugation.

After iron removal, excess radioactive ferric ammonium citrate and then sodium bicarbonate solution are added to the serum solution. Excess iron is removed with Amberlite IRP-67 and CG-120 Type-3 powder. By this method, TIBC can be rapidly and directly determined.

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