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Coated Charcoal Assay of Plasma Iron Binding Capacity and Iron Using Radioisotope Dilution and Hemoglobin-Coated Charcoal[1,2]

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INTRODUCTION

In vitro assays of a number of biologic constituents have recently been described, in which the free agent was rapidly and essentially completely separated from its bound form by batch separation with coated charcoal (2-8). Using this technique, the unsaturated iron binding capacity (UIBC) of plasma can be assayed by adding $^{59}FeCl_3$ to plasma and removing the excess unbound radioiron from the iron-transferrin complex by charcoal coated (saturated) with hemoglobin, thereby permitting determination of UIBC from the quantity of ^{59}Fe remaining in solution (1,6). In the present communication, we explored the use of the coated charcoal technique and the principle of radioisotope dilution for assay of plasma iron. The native iron bound to transferrin in plasma was released by lowering the pH of the plasma. This diluted the specific activity of

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a known quantity of subsequently added $^{59}FeCl_3$. Restoration of the physiological pH of the plasma permitted rebinding by transferrin of a portion of the pool of native iron mixed with radioiron. The excess free iron (native and $^{59}FeCl_3$) was then removed by coated charcoal. This series of events is schematically depicted in Figure 1. The plasma iron level was then obtained from a simple equation.

MATERIALS AND METHODS

Plasma

Plasma samples were collected in iron-free heparinized[1] tubes and stored at −20° C.

Buffers

Four-tenths molar phosphate buffer, pH 5.3, with iron content of less than 0.003 per cent for the stock powder, and 0.4 molar tris-hydroxy methylamino methane-hydrochloride (TRIS-HCl) buffer, pH 9.0, were prepared.

Iron

Working solutions containing 6 micrograms of elemental iron per ml of deionized water were prepared using ferrous ammonium sulfate as previously described (6). Radioactive iron chloride ($^{59}FeCl_3$) was purchased from Oak Ridge National Laboratory in lots of 1,000 microcuries with a specific activity of 10 to 40 microcuries per microgram. A working solution of radioiron containing 6 micrograms per ml of elemental iron labeled with tracer amounts of $^{59}FeCl_3$ was prepared as previously described (6).

Hemoglobin-coated charcoal

This is prepared by mixing equal volumes of a 5 gram per cent aqueous suspension of Darco, grade HDB (Hydrodarco B) activated carbon[1] and a 0.5 gram per cent aqueous solution of hemoglobin derived from outdated human bank blood (3,6). The use of one part hemoglobin to ten parts Hydrodarco B charcoal by weight permits essentially instant separation of 97.6 to 99.5 per cent of free iron from iron bound to transferrin. Other charcoals with similar particle size, such as Norit A neutral pharmaceutical grade charcoal (6), do not provide adequate separation of free from transferrin-bound iron. As much as 15 per cent of the free iron may not be adsorbed by Norit A charcoal in the present assay method. This appears to relate to the introduction of phosphate buffer into the procedure, since excellent separation of the free from bound mineral may be achieved by hemoglobin-coated Norit A charcoal in the assay for plasma UIBC, in which phosphate buffer is not required (6).

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[1]Vacutainer No. 3200 KA, purchased from Becton, Dickinson and Co., Rutherford, New Jersey. All glassware must be scrupulously clean, because contamination with iron voids the assay.

Assay protocol

To duplicate samples in 10 ml test tubes containing 0.5 ml of unknown plasma (pH usually 7.6 to 8.1 on storage) for assay is added 0.5 ml of 0.4 molar phosphate buffer, pH 5.3, which reduces the pH of the plasma to approximately 5.8, a number low enough to cause the dissociation of iron from transferrin *in phosphate buffer*. The contents of the tubes are mixed well and incubated at 37°C for 30 minutes to insure complete separation of the native iron from its transport protein. One-half ml of ferrous ammonium sulfate solution labeled with $^{59}FeCl_3$ containing 6 micrograms of elemental iron per ml is added and the contents again mixed well. One-and-one-half ml of 0.4 molar TRIS-HCl buffer, pH 9.0, is then added, raising the pH of the mixture to approximately 7.5 (range 7.4 to 7.9), and the contents of the tubes are mixed well and incubated at 37°C for 30 minutes to allow saturation of the plasma transferrin by the mixture of unknown native iron and radioactive iron. Two ml of hemoglobin-coated Hydrodarco B charcoal suspension are then added and the tubes are capped with Parafilm and mixed by inverting five times. The tubes are then centrifuged at 3,000 rpm for 15 minutes to sediment the charcoal containing

[1]Darco, grade HDB was supplied by Atlas Chemical Industries, Inc., Wilmington, Delaware, and is made from lignite by heat activation. This charcoal is used for treatment of municipal water supplies; a 50 lb. bag costs $7.50; one pound is enough for 9,080 coated charcoal iron assays. Fifty per cent of the particles are less than 15 microns in diameter and 30 per cent are between 10 and 2 microns. The pH of a water extract ranges between 9 and 11 (9).

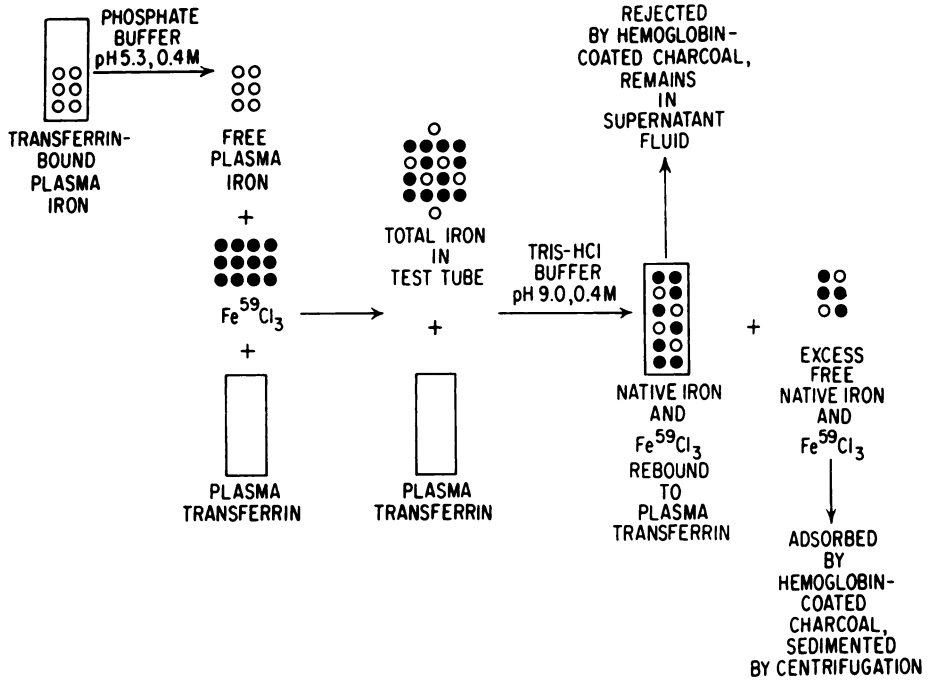


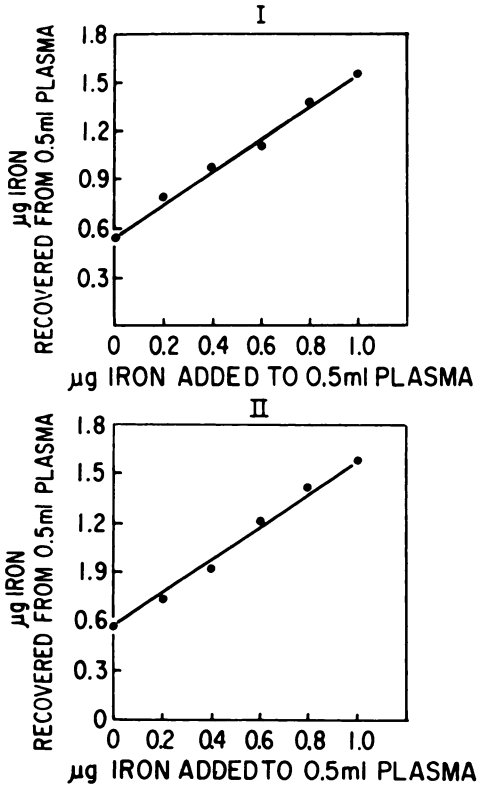
Fig. 1. Schematic depiction of the coated charcoal assay for plasma iron level.

excess free iron. The supernatants containing transferrin-bound iron are decanted and their radioactivity determined in a well-type scintillation counter.

A supernatant control consisting of deionized water in place of plasma, and a standard containing 0.5 ml (3 micrograms) of the working solution of radioactive iron and 4.5 ml of deionized water without charcoal are run with each group of unknowns. In addition, duplicate 0.5 ml unknown plasma samples are assayed concurrently to determine their unsaturated iron-binding capacity (UIBC) under the conditions of the procedure. This is necessary for accurate calculation of the plasma iron level. In this procedure the TRIS-HCl buffer is added prior to the radioiron solution. The sequence of addition of reagents is: unknown plasma, phosphate buffer, TRIS-HCl buffer, radioiron and hemoglobin-coated charcoal.

Calculation of plasma iron

The counts per minute (cpm) for the supernatant control, which represents the excess free iron not cleared from the supernatant by hemoglobin-coated charcoal (generally, one to two per cent of the added iron) are subtracted from the cpm of the unknown plasma and from the control UIBC, respectively, in



(μg) IRON ADDED	(μg) IRON RECOVERED I	II
	0.54	0.57
0.2	0.78	0.74
0.4	0.97	0.93
0.6	1.12	1.21
0.8	1.37	1.41
1.0	1.56	1.58

Fig. 2. Recovery curves. Increments of 0.2 micrograms of cold iron were added to 0.5 ml of plasma containing 0.55 micrograms of native iron, and assayed two weeks apart.

order to obtain net cpm. The plasma iron level is calculated from this formula:

$$\mu g\ Fe/0.5\ ml\ plasma = B/F\ (\mu g\ Fe^{59}\ added - \mu g\ UIBC) - \mu g\ UIBC$$

where B = net cpm of the transferrin bound radioiron of the unknown sample (i.e., B = cpm in supernatant).

F = the difference between the net cpm added (standard) and B, i.e., free radioiron (i.e., F = S − B, when S is cpm of the standard)

μg added = the amount (3.00 μg) radioiron added in the assay, and

μg UIBC = the net cpm of the control UIBC run concomitantly in the assay and expressed as μg by reference to the standard.

Derivation of the formula.

$$\text{Specific Activity (SA)} = \frac{\text{counts per minute (cpm)}}{\text{weight } (\mu g)} \quad \ldots\ldots (1)$$

Thus by radiodilution:

$$\text{SA supernatant (bound Fe)} = \text{SA coated charcoal pellet (free Fe)} \quad \ldots\ldots (2)$$

since:

$$\text{SA supernatant} = \frac{\text{cpm supernatant}}{\mu g \text{ plasma Fe} + \mu g \text{ UIBC}} \quad \ldots\ldots (3)$$

and:

$$\text{SA charcoal} = \frac{\text{cpm charcoal}}{\mu g \text{ added radioiron} - \mu g \text{ UIBC}} \quad \ldots\ldots (4)$$

but,

$$\text{cpm charcoal} = \text{cpm added radioiron} - \text{cpm supernatant} \quad \ldots\ldots (4a)$$

then:

$$\text{SA charcoal} = \frac{\text{cpm added radioiron} - \text{cpm supernatant}}{\mu g \text{ added radioiron} - \mu g \text{ UIBC}} \quad \ldots\ldots (5)$$

substituting for (2):

$$\frac{\text{cpm supernatant}}{\mu g \text{ plasma Fe} + \mu g \text{ UIBC}} = \frac{\text{cpm added radioiron} - \text{cpm supernatant}}{\mu g \text{ added radioiron} - \mu g \text{ UIBC}} \quad \ldots\ldots (6)$$

solving for μg plasma Fe:

$$\mu g \text{ plasma Fe} = \frac{\text{cpm supernatant } (\mu g \text{ added radioiron} - \mu g \text{ UIBC})}{\text{cpm added radioiron} - \text{cpm supernatant}} - \mu g \text{ UIBC} \quad \ldots (7)$$

but,

$$\text{cpm supernatant} = \text{bound radioiron (B)} \quad \ldots\ldots (7a)$$

and,

$$\text{cpm added radioiron} - \text{cpm supernatant} = \text{free radioiron (F)} \quad \ldots\ldots (7b)$$

then:

$$\mu g \text{ plasma Fe}/0.5 \text{ ml} = B/F\ (\mu g \text{ added radioiron} - \mu g \text{ UIBC}) - \mu g \text{ UIBC} \quad (8)$$

Comparison with an established method

All unknown plasma samples assayed by the coated charcoal method were assayed for plasma iron level and UIBC by colorimetric methods for comparison (10,11).

RESULTS

Recovery of known amounts of added cold iron

Figure 2 shows the excellent recovery of known amounts of nonradioactive iron added to plasma using the coated charcoal assay. The assays were performed using 0.5 ml of plasma to which was added increments of 0.2 micrograms of nonradioactive iron. Two graphs (I and II) are presented in the figure to demonstrate the reproducibility of the assay system using a single plasma source. The second assay was performed two weeks after the first.

Linear relation

Figure 3 shows two radiodilution curves replotted from the data in Figure 2. Similar curves have been described for assay of other biologic constituents using the coated charcoal technique (3,4), as well as for a number of hormones determined by radio-immunoassay as described for insulin by Yalow and Berson (12). In such systems the binder specific for the substance being assayed was not normally present in the plasma (serum) to be assayed.

Figure 4 shows the data from Figure 3 replotted, replacing the ordinate ratio of bound/free ^{59}Fe with B/B′, where B equals the cpm of ^{59}Fe represented by the total iron binding capacity of the plasma and B′ equals the cpm of ^{59}Fe diluted by iron native to and added to the plasma bound by the same quantity of transferrin. The plots along the abscissa represent the sums of the added cold iron and the iron native to the plasma. The plots of observed values from both experiments fall close to anticipated values (solid line); the latter is obtained by extrapolating the line (interrupted in the figure) connecting the added radioactivity and the ratio of 1 on the ordinate for the TIBC of plasma with undiluted radioiron.

Figure 5 compares the plasma iron levels of 105 consecutive subjects assayed independently by the Ramsey (R-values) and coated charcoal (CC-values) methods. The assays include the entire spectrum of plasma iron levels from marked hypoferremic to marked hyperferremic states. The UIBC of the plasma was assayed simultaneously as a control. The TIBC of the plasma showed the expected considerable variation between patients. The amount of labeled iron was constant in the entire series and was fixed at 3.0 micrograms per 0.5 ml of plasma. The R and CC values have the sample correlation coefficient $r = 0.776$, which is highly significant for $n = 105$. The 95 per cent confidence limit for the corresponding r_p (population correlation coefficient) is given

by $r_{p_1} = 0.84$ and $r_{p_2} = 0.68$. The solid line in Figure 5 represents the regression line of R on CC and is given by the equation:

$$R = 0.94\ CC - 2 \text{ (where all values are given in } \mu C/100 \text{ ml)}$$

The corresponding error of estimate is 39. That means that if it can be assumed that R is distributed normally about the line of regression, its value associated with a given CC will, in 68 per cent of cases, lie between $R_1 = 0.94\ CC + 37$ and $R_2 = 0.94\ CC - 41$, i.e., between the two broken lines in Figure 5. Actually, as can be seen from Figure 5, more than 68 per cent of all cases fall within these limits.

Table 1 shows the raw data and calculated results for the plasma iron level and control UIBC level for 10 unknown samples, assayed after the technic had been fully worked out and after the 105 samples in Figure 5 were assayed

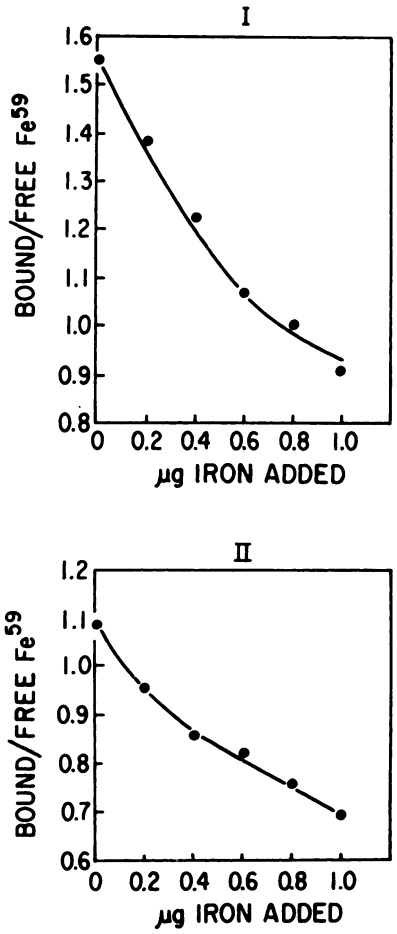


Fig. 3. Replot of the data from Figure 2, replacing the ordinate values with the ratio of bound to free Fe^{59}.

(i.e., when experience with the method was sufficiently great that results should be expected to be at their best). The results are compared with those obtained by the colorimetric methods. In general, for plasma iron level, duplicates varied by 1 or 2 per cent with an occasional variation up to 5 per cent; for the control UIBC level, duplicates varied from 1 to 5 percent. The control UIBC level parallels the UIBC level of the plasma obtained by the method of Schade, and by the previously reported UIBC method (6).

DISCUSSION

In the present method, the binder (transferrin) for the unknown (iron) is intrinsic to the assay medium (plasma). The iron binding capacity of the uncomplexed transferrin (total iron binding capacity, TIBC) must be preserved during the release of native iron for radiodilution so that sufficient binding capacity remains to bind a portion of the pool of free iron (native and radioactive). In normal subjects the TIBC of plasma ranges from 225 to 475 micrograms per 100 ml (13), with narrower limits reported as 280 to 360 micrograms per 100 ml (14). For our laboratory the normal values are: plasma iron 80 to 150 micograms per 100 ml; TIBC 240 to 400 micrograms per 100 ml. In a variety of chronic diseases (inflammatory, infectious and neoplastic) and in hemochromatosis the TIBC of plasma is moderately reduced. Rarely, the TIBC of plasma may be severely reduced (15). In iron deficiency and hepatitis the TIBC of plasma may be moderately increased. In pregnancy, the total binding capacity may be considerably increased (particularly with coincident iron deficiency) as has been reported in pregnancy for the transport protein of hormones, and for vitamin B_{12} binding proteins (16). Thus, with the addition of a fixed amount of radioiron (3.0 micrograms) and a variable TIBC dependent on the underlying condition of the patient, the sensitivity of the assay will vary, tending to be most sensitive when the "biopsy" of the diluted radioiron is large (normal or increased TIBC) and less sensitive when the "biopsy" is small (decreased TIBC). In the latter instance, increased sensitivity may be obtained by using more plasma or less radioiron.

In 1953, Feinstein, *et al* (17), suggested the use of radioisotope dilution to measure the amount of protein-bound serum iron. They suggested that native iron which may be released from its binder by reducing the pH of the serum could be used to dilute the specific activity of a known quantity of subsequently added radioiron. Returning the pH of the medium to normal would permit the protein to recombine with iron to the point of saturation. They planned to use neutralized saturated ammonium sulfate to precipitate the protein-bound iron and then determine the excess free radioiron in the filtrate, but never completed that study. The recent use of coated charcoal to adsorb free substances and reject them when bound to their transport protein or antibody suggested a number of assay procedures. The basic requirements for the coated charcoal assay to be applicable for a specific determination have been previously reported (3), and include: (1) adsorption by coated charcoal of the free agent; (2) rejection by coated charcoal of the agent when complexed with its binder; (3) availablity of a marker (radioactive or other) for the agent.

Fifty mg of coated charcoal affords essentially instantaneous separation of free iron from transferrin-bound iron, when 0.5 ml of plasma is under study. In the absence of plasma proteins approximately 98 or 99 per cent of the added radioiron is cleared by hemoglobin-coated charcoal. In the presence of plasma proteins a small quantity of radioiron (1 to 5 per cent) appears to be loosely bound to albumin and possibly other proteins, nonspecifically (6). Thus, a small error is introduced when a saline supernatant control is used in the assay rather than a standard iron-saturated plasma supernatant control (sequence of addition of reagents identical to the coated UIBC). As such, the observed experimental value will be slightly lower than the true value, which for practical purposes is inconsequential.

The major source of stable iron contamination other than improperly cleaned glassware and poor technique is the trace of iron present in the buffer salts. The TRIS-HCl buffer contains a negligible amount of iron contamination (less than 4 micrograms per cent). The use of appropriate phosphate buffer will usually add less than 10 micrograms per cent iron to the system. However, occasionally as much as 40 micrograms per cent iron has been noted. The

I (•)		II (x)	
B/B'	cpm Fe^{59}	B/B'	cpm Fe^{59}
1.000	10,653	1.000	21,342
1.178	9,044	1.189	17,949
1.234	8,632	1.269	16,824
1.303	8,173	1.340	15,929
1.388	7,673	1.375	15,524
1.432	7,441	1.438	14,837
1.507	7,069	1.517	14,065

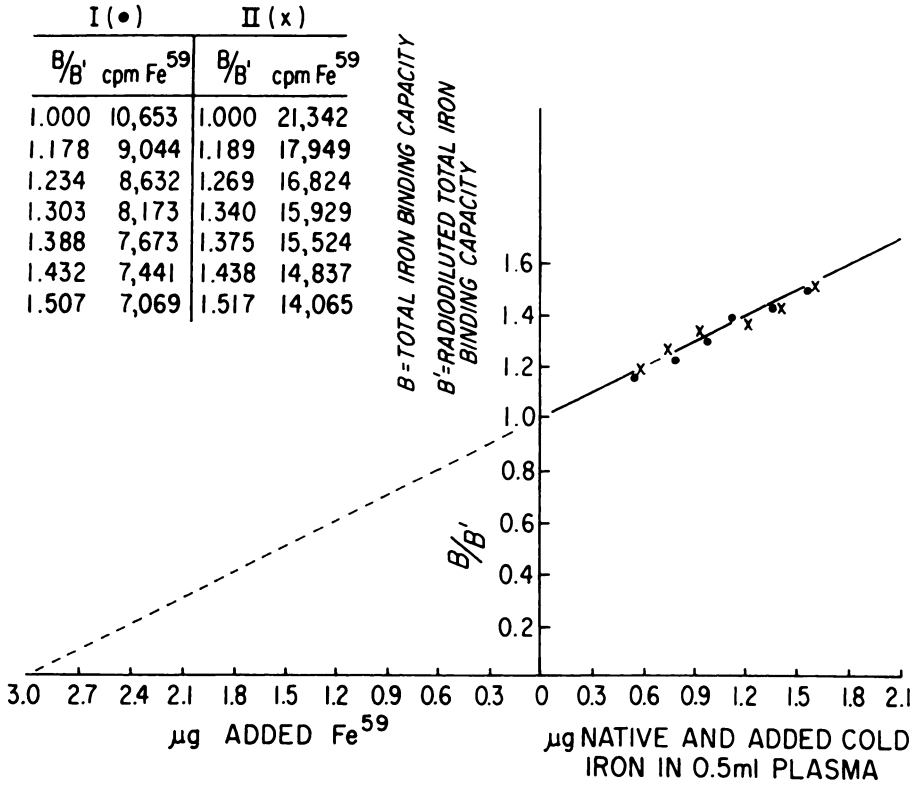


Fig. 4. Replot of the data from Figure 3 replacing the ordinate with B/B', where B equals the total iron binding capacity and B' equals the radiodiluted total iron binding capacity. The native plasma iron level and increments of added iron are plotted along the abscissa.

amount of iron in this buffer solution should be determined to assure reasonable freedom from contamination. Using the coated charcoal assay (6), the iron content may be assessed by noting the reduction in the UIBC of a standard plasma by introducing the phosphate buffer into the system prior to the addition of radioiron. Care should be taken to use buffers of the same ionic concentration for comparative results, since the UIBC of plasma tends to decrease with increasing molarity of the buffer (6,18). If the iron content of the phosphate buffer is known and a significant quantity (arbitrarily more than 10 micrograms per cent for the 0.4 molar buffer), this value can be subtracted directly from the final plasma iron level measured in the assay. Alternatively, iron-free phosphate buffer may be prepared (11).

The control UIBC of plasma provides experimental identity of reagents for the final computation of the plasma iron level. It offers the added advantage that it closely parallels the UIBC of plasma obtained by the spectrophotometric method. Occasionally, for unknown reasons, there may be moderate differences, generally in that the control UIBC is reduced compared to the UIBC obtained by standard techniques. For greater reliability the previously described

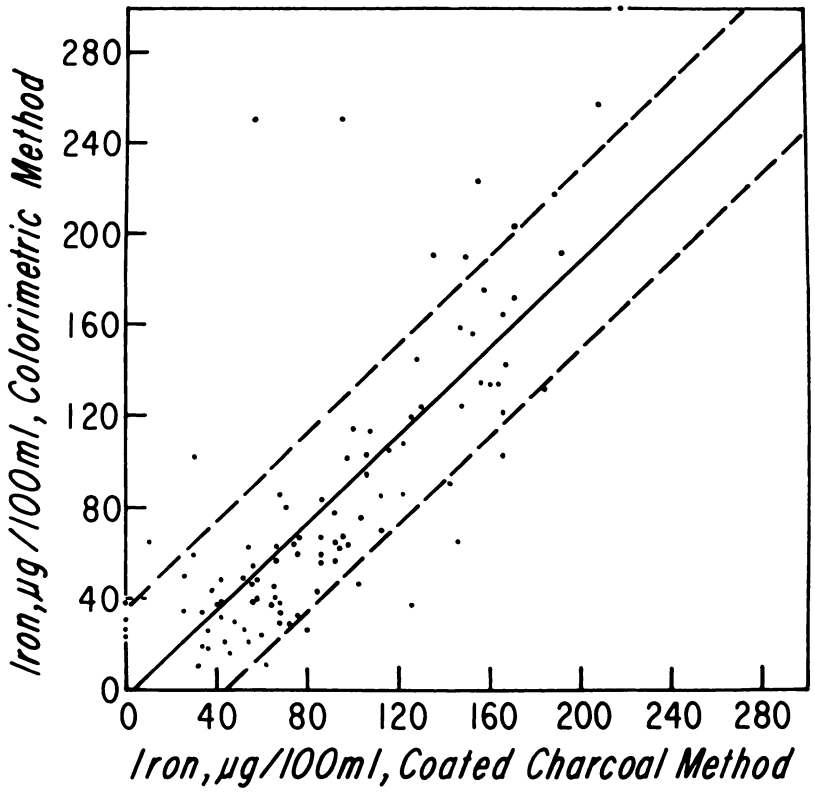


Fig. 5. Comparison of plasma iron levels in 105 subjects by the Ramsey vs. the coated charcoal method.

method (6) for assessing the UIBC of plasma is preferable, although the method described herein may provide satisfactory screening of the UIBC.

The coated charcoal assay provides an acceptable alternative to the colorimetric assays now available, with the added advantage that icteric, hemolyzed, and lipemic plasmas may be evaluated (6). In addition, the present system provides a model for the assay of agents in which the binder is naturally present in the biologic fluid under assay. An alternative approach in which the binder is completely destroyed (but the agent preserved) so that rebinding of the agent may be accomplished by utilizing a standard, similar medium with a preselected binding capacity for the agent, as for serum vitamin B_{12} assay (3), was not successful in the case of transferrin. Despite prolonged heating (greater than 1 hour at 100°C in a water bath) in the presence of strong acid (0.25 N HCl) we were unable to completely destroy the intrinsic transferrin-binding capacity for iron (or other nonspecific iron binder) of the plasma

TABLE I

RAW DATA, COATED CHARCOAL PLASMA IRON ASSAY

Patient	*Av. net cpm Fe^{59} in Supernatant*[1]		*Plasma Iron (μg/100 ml)*		*Plasma UIBC (μg/100 ml)*	
	Iron	*UIBC*[1,2]	*Coated Charcoal Method*	*Ramsey Method*	*Coated Charcoal Plasma Iron Method*[2]	*Coated Charcoal UIBC Method*[3]
M.W.	607 ± 7	354 ± 1	182	213	148	148
M.S.	1171 ± 23	1157 ± 10	34	26	484	502
C.L.	729 ± 1	684 ± 15	40	37	286	302
S.K.	421 ± 7	330 ± 8	56	51	138	154
A.S.	330 ± 2	233 ± 4	52	43	98	106
D.M.	610 ± 8	526 ± 9	62	61	220	250
N.N.	671 ± 1	637 ± 4	28	32	266	280
D.H.	376 ± 3	114 ± 2	150	192	48	30
G.C.	607 ± 23	543 ± 18	46	50	228	278
C.J.	669 ± 24	604 ± 6	52	69	252	294
Supernatant Control[4]	35 ± 1	(2.44%)[5]				
Standard[6]	1433 ± 13 (cpm per 3 μg Fe^{59})					

[1]Obtained by subtracting the supernatant control cpm from the average of the duplicate cpm.

[2]Represents the unsaturated iron binding capacity (UIBC) obtained when phosphate and tris hydroxy methylamino methane hydrochloride buffers are used in the system for assay of plasma iron level.

[3]As published in reference 6. Results with Schade method were essentially identical.

[4]In this control, 0.5 ml of plasma is replaced with 0.5 ml of deionized H_2O.

[5]Represents the unbound Fe^{59} remaining in the supernatant after treatment with coated charcoal.

[6]Represents the cpm per 3 μg of Fe^{59} in a volume of 5 ml.

under study. Similar treatment destroys alpha and beta B_{12} binding proteins in plasma, with seeming preservation of the vitamin B_{12} molecule, so that binding of a radio-diluted pool of vitamin B_{12} by an extrinsic binder (intrinsic factor in this instance) can be accomplished (3).

SUMMARY

Using radioisotope dilution, and hemoglobin-coated charcoal for batch separation, plasma iron may be measured. From 0.5 ml of plasma, native transferrin-bound iron is released at pH 5.8 (by adding 0.5 ml of pH 5.3 buffer to the plasma), following which 3 μg labeled iron is added. After subsequent addition of buffer to raise the pH to 7.4 and thus allow rebinding of a portion of the pool of radiodiluted iron by transferrin, iron-transferrin complex is separated from excess free iron by hemoglobin-coated charcoal and the plasma iron level computed by an appropriate formula. This method provides a model for the assay of similar constituents in biologic fluids, in which the binder for the agent is naturally present in the fluid under assay.

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SYMPOSIUM ON RADIOISOTOPES METHODOLOGY

September 30 & October 1, 1967

Palmer House, Chicago, Ill.

During the 1967 Annual Meeting of the American Society of Clinical Pathologists a special two-day (Saturday and Sunday) Radioisotopes Symposium, cosponsored by the Society of Nuclear Medicine, will be conducted for the participation of Laboratory Physicians and their Technologists.

The Symposium is designed both for those interested in setting up laboratory tests involving radioisotopes and for those desiring details of theoretical aspects behind certain tests. Speakers will be both laboratory physicians and their technologists. The former will describe the broad background of many commonly used clinical tests, the latter will present "brass tacks" lectures on exactly how to perform the various tests and how the performance relates to the theory behind them. Among the general topics to be covered are: Thyronine Protein Complexes, Iron Protein Complexes, Vitamin B-12 Protein Complexes, Renal Function, Radioimmunology.

The registration fee for the two-day Symposium is $25.00 which includes a Manual distributed at the exercise. The ASCP Radioisotopes Symposium is supported in part by a grant-in-aid from the Radio-Pharmaceutical Division of Abbott Laboratories. Please send registration request, together with check payable to the American Society of Clinical Pathologists, to the Society's Headquarters: 445 North Lake Shore Drive, Chicago, Illinois 60611.