compete with Fe$^{2+}$ for binding sites (9), it is imperative that reagents and glassware be made metal-free.

In vitro techniques involving iron as a reactant should avoid the use of synthetic iron chelators of the EDTA, citrate, or oxalate types, if the intent is to bind iron to a protein or incorporate it into a macromolecule (11, 12).

Using lyophilization vials and conducting the reaction under a positive pressure of N$_2$ and anaerobic conditions helped to promote the reaction. Freshly prepared ascorbic acid assured that its full reduction potential would be available to keep iron in the ferrous state (13).

In summary, we optimized reaction conditions and developed a radiochemical procedure for measuring ferrochelatase, which, because of its sensitivity through use of $^{59}$Fe with high specific activity and minimization of O$_2$ content of the reaction, should find great utility in the measurement of this enzyme in other tissues. The greater sensitivity allows use of smaller sample sizes or shorter incubation times, or both. Preliminary studies in this laboratory indicate that this procedure is adaptable to measurement of ferrochelatase in bone marrow and reticulocyte-rich lysates.

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CLIN. CHEM. 26/1, 156–158 (1980)

Use of An Alumina Column in Estimating Total Iron-Binding Capacity
Ross T. Starr

I describe a simple, rapid technique for saturating the transferrin in serum and then removing unbound (excess) iron. This technique involves use of an easily prepared column of basic chromatographic alumina and a saturating solution of ferric chloride in citric acid. This method, when compared with the magnesium carbonate method of Ramsay (Clin. Chim. Acta 2: 221, 1957) by regression analysis and tests of precision, showed a negative bias for results by the alumina technique with respect to the magnesium carbonate method.

Estimation of iron-binding capacity of serum has an established role in the diagnosis of such conditions as iron-deficiency anemia and hemochromatosis. The determination of total iron-binding capacity (TIBC) has remained an imprecise, time-consuming manual technique, despite the many technical advances in serum iron estimation.

TIBC techniques currently in use include the saturation of transferrin and removal of excess iron from serum with an adsorbent such as magnesium carbonate (1) or an ion-exchange resin (2). Other techniques rely on measurement of the excess iron after saturation and calculation of unsaturated iron-binding capacity (3, 4) and various radiometric techniques. Of the adsorbent techniques used in continuous-flow analysis, atomic absorption spectrophotometry, and some manual colorimetric iron methods, the magnesium carbonate method of Ramsay (1) is one of the more common.

The method presented here has advantages over the magnesium carbonate method in ease of handling and speed while offering a closer agreement with the lower values for TIBC encountered with the non-adsorbent techniques.

Materials and Methods

Apparatus

Spectrophotometry: All spectrophotometric measurements were made with a Coleman 55 spectrophotometer (Perkin-Elmer Corp., CT 06852).

Columns: Surety Columns No. 1 (Evergreen Scientific, Los
Fig. 1. Comparison of results for 102 sera by the present method and the MgCO₃ method

y-intercept = 7.2, slope = 0.93, r = 0.97, mean x(±SD) = 60.0 ± 15.7, mean y = 63.2 ± 15.0, standard error of regression = 3.6 μmol/L.

Fig. 1. Comparison of results for 102 sera by the present method and the MgCO₃ method

Angeles, CA 90058). These columns are designed to fit over AutoAnalyzer cups and filter out any particles.

Reagents: Basic aluminum oxide (alumina, Brockman Grade II), ferric chloride, citric acid (all from BDH Chemicals, East Melbourne, Victoria 3002, Australia).

Procedures

Preparation of columns: About 0.8 g of dried alumina is dispensed into the Evergreen columns, which are then placed above AutoAnalyzer cups or some other receptacle.

Some batches of alumina contain fines, which may pass through the mesh support when fluids are applied to the column. To overcome this problem, wash the alumina as follows. Add a quantity of alumina to about five times its volume of distilled water in a conical flask. Swirl the flask and allow it to stand for about 1 min before decanting. Repeat this five times, or until the alumina settles rapidly. Then dry the alumina in a hot-air oven.

Preparation of Saturating Solution

Stock solution: Prepare 500 mg of Fe³⁺ (as ferric chloride) per liter of 0.1 mol/L citric acid. This solution is stable at least three months if stored at 4 °C.

Working solution: Dilute the stock solution 100-fold in distilled water. Prepare freshly each week.

Saturation and adsorption test: Add 2 mL of working solution to 1 mL of serum in a test tube, allow to stand for 3 to 5 min, and pour onto the column; after 6 or 7 min the mixture will have passed through the column and is ready for iron assay.

Iron Estimation

Iron estimations on the treated sera for both the proposed method and the magnesium carbonate method were performed by the manual bathophenanthroline ICSH method (5).

Results

Regression Analysis: Al₂O₃ vs MgCO₃

TIBC was estimated by the magnesium carbonate method and the proposed alumina method on 102 specimens received in our laboratory (Fig. 1).

Precision

Within-run precision was assessed by use of two pooled specimens of human serum and one lyophilized bovine control serum (Table 1).

Between-run precision was assessed by use of a pooled specimen human serum pool and a bovine control serum, both stored frozen (Table 2).

Efficiency of Adsorption

To investigate the efficiency of adsorption of iron by the alumina, I treated 1-mL aliquots of serum pool with 2-mL volumes containing increasing concentrations of iron and then passed them through alumina columns. Aliquots of the same serum pool were treated in the same way by the magnesium carbonate method. Iron estimates were then carried out and the results, plotted as TIBC vs added iron, showed curves for both methods that nearly coincide, plateauing at about 4 μg of added iron per liter. The iron in the serum pool was 9.7 μmol/L.

Of interest here is the disparity between the two methods until the saturation point is reached. As would be expected, the TIBC of the blank (no added iron) of the alumina series is near the iron value for the serum pool. However, the blank for the magnesium carbonate series is considerably higher. Subsequently it was shown that the magnesium carbonate in use contained residual iron, which the transferrin was apparently sequestering.

Cook (7) has observed an adsorption inefficiency with magnesium carbonate and other adsorbents, in that measured TIBC increases with increases in added iron over that which is required to saturate the transferrin. This effect has been observed on some occasions when iron was measured by the ICSH method. The same effect has not been observed with the alumina method. My results show that the alumina is at least equal to magnesium carbonate in adsorption efficiency.

Up to 1.5 μg of added iron, the slopes of the lines in the above-mentioned plot were very similar, and if the blank iron-binding capacity was subtracted from the 1.5-μg value

Table 1. Within-Run Precision

<table>
<thead>
<tr>
<th></th>
<th>Human pool I</th>
<th>Human pool II</th>
<th>Bovine lyophilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Al₂O₃</td>
<td>MgCO₃</td>
<td>Al₂O₃</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>TIBC mean, μmol/L</td>
<td>52.5</td>
<td>59.5</td>
<td>56.1</td>
</tr>
<tr>
<td>SD</td>
<td>1.29</td>
<td>1.81</td>
<td>0.80</td>
</tr>
<tr>
<td>CV, %</td>
<td>2.5</td>
<td>3.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* The iron estimations for this set were done by continuous-flow, by a modification of the method of Giovanniiolo et al. (6).

Table 2. Between-Run Precision

<table>
<thead>
<tr>
<th></th>
<th>Human pool</th>
<th>Bovine control serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Al₂O₃</td>
<td>MgCO₃</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>TIBC mean, μmol/L</td>
<td>51.5</td>
<td>55.8</td>
</tr>
<tr>
<td>SD</td>
<td>2.22</td>
<td>2.97</td>
</tr>
<tr>
<td>CV, %</td>
<td>4.3</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* The stated value for TIBC for the bovine control serum for manual iron methods involving MgCO₃ was 56.7–59.7 μmol/L.
iron-binding capacity, the corresponding TIBC values were
27.0 μmol/L by the alumina method and 26.1 μmol/L by the
magnesium carbonate method, or, in terms of analytical re-
covery, 100.5% for alumina and 97.2% for magnesium car-
bonate.

Discussion

From the data it is apparent that the proposed alumina
method has a negative bias with respect to the magnesium
carbonate method.

After centrifugation of the magnesium carbonate many fine
particles of magnesium carbonate can be seen to remain sus-
pended in the supernate. These particles probably contain
some iron, which is measured along with transferrin-bound
iron. Cook (7) noted a considerable decrease in TIBC after
repeated and prolonged centrifugation. He has also noted
the above-mentioned “adsorption inefficiency” with magnesium
carbonate. This effect could not be demonstrated with alu-
mina.

Ramsay (8) has shown the magnesium carbonate method
to give results that are 1–10% high, owing to inclusion of
non-transferrin-bound iron in the supernate.

Another factor contributing to the bias is an opacity effect,
which is produced at the protein-precipitation stage of the
ICSH iron method with some sera that have been treated by
the magnesium carbonate method. This opacity is carried
through the color-development stage and in some cases makes
a spectrophotometric reading impossible. This effect is often
found in patients with abnormal serum protein concentra-
tions. In most cases the alumina method does not generate any
opacity, although some opacity will occasionally be found. The
opacity effect occurs most often among sera of lower TIBC,
and may have contributed to a reduced slope and increased
y-intercept in the regression equation.

Among the 102 paired measurements in the regression
analysis of alumina (x) vs magnesium carbonate (y), there
were 16 pairs whose difference (y – x) exceeded twice the
standard error of regression. Of these 16, 12 had x values below
the mean value of x. If these 16 pairs are rejected and the re-
gression analysis repeated, the equation (Figure 1) becomes:
y = 4.88 + 0.95x (r = 0.98, n = 86).

The Burroughs Wellcome Quality Control Programme on
lyophilized bovine sera indicates a positive bias of magnesium
carbonate and other adsorbent methods with respect to the
non-adsorbent techniques for TIBC determination. Although
the limits of bovine sera are well known, no such extensive
comparison has, to my knowledge, been made with human
sera.

I saw no departures from the normally found differences
in TIBC between the magnesium carbonate and alumina
methods with lipemic, icteric, and hemolysed sera.

The alumina method may be carried out in a centrifuge
tube, substituting for use of the column a 10-min mixing stage
on a rotator followed by centrifugation. This technique would
not require the alumina to be washed, but for the sake of
uniformity all alumina should be dried to Brockman Grade I.

In summary, I believe the alumina method offers advan-
tages over the magnesium carbonate method in ease of han-
dling and speed while offering a closer agreement with the
lower values for TIBC encountered with the non-adsorbent
techniques.

I thank Dr. G. B. Leyton for his advice and help, C. Bradshaw for
technical assistance, and N. Balaza and J. Heath for help with the
continuous-flow work.

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