Clinical Determination of Methemalbumin

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We developed an assay for methemalbumin in biological fluids by using diethylaminoethyl-Sepharose ion-exchange chromatography to separate this protein from interfering components, including hemopexin, transferrin, hemoglobin, and haptoglobin/hemoglobin complex. Initial screening of the samples requires measurement of $A_{280}/A_{405}$ ratios of the peak tubes of the isolated albumin fraction. Values exceeding 30 indicate that methemalbumin is absent, and no further work is required. Values of less than 30 suggest that methemalbumin is present in the original sample, whereupon the presence and amount of methemalbumin can be ascertained by colorimetric assay for iron with use of ferrozine. Results may be expressed either in terms of micrograms of methemalbumin iron per gram of albumin or in milligrams of methemalbumin per liter. The reproducibility of the method is of the order of ±7% (SD). Normal persons have essentially no methemalbumin iron in their serum. Three individuals with hemorrhagic pancreatitis showed values of 65, 98, and 198 μg of methemalbumin Fe per gram of albumin.

Additional Keyphrases: biological fluids • chromatography on DEAE-Sepharose • iron content as a measure of methemalbumin • hemolytic and hemorrhagic disorders • pancreatitis

Certain hemolytic and hemorrhagic disorders result in accumulation of methemalbumin in serum (1-3). Determination of this constituent may be of great value, particularly in differential diagnosis of acute vs. hemorrhagic pancreatitis (4-10). Existing assay methods rely on direct spectrophotometric analysis of whole serum (11, 12), a technique in which somewhat arbitrary correction factors have to be used. Although at least one of these methods (13) appears to give clinically valid data, we wished to devise a relatively uncomplicated separation scheme that would lead to a definitive assay of methemalbumin in biological samples.

The method developed depends on the chromatographic isolation of methemalbumin from other iron-containing compounds such as transferrin, methemoglobin, and hemoglobin. The methemalbumin content is estimated by measuring the ratio of the absorbances at 280 and 405 nm of an appropriate eluate fraction. The first gives an estimate of albumin content, the second of heme-containing compounds. Normally this ratio will exceed 30. If it is <30, iron is determined quantitatively in the same eluates by a modification of a method used to measure serum iron (14, 15), and the results are expressed in micrograms of iron per gram of albumin. Values of 0 to 5 μg/g are considered normal. Values exceeding 20 μg/g are indicative of the presence of methemalbumin in the initial sample.

A specimen can be screened in 45 min; a definitive quantitative workup, including the iron assay, can be done in 2 h.

Materials and Methods

Apparatus

For the colorimetric iron assay we used a double-beam grating spectrophotometer (Model 124; Coleman Instruments, Oakville, Ill. 60521) set at λ_max = 562 nm. The same instrument was used to monitor protein elution from the column at λ_max = 280 nm, as well as heme elution at λ_max = 405 nm.

The columns used to separate the methemalbumin were home-made glass columns (i.d., 1.27 cm; height, 8 cm) fitted with rubber stoppers at both ends, which in turn were connected to polyethylene tubing (i.d., 0.11 cm).

A Model 17000 Minirack Fraction Collector (LKB Instruments, Inc., Rockville, Md. 20852) was used to collect the column eluate.

To minimize contamination, disposable culture tubes and a sampler (Oxford Labs., Foster City, Calif. 94404) were used for all steps of the iron assay.

Reagents and Stock Solutions

Diethylaminoethyl-Sepharose (DEAE-Sepharose A-50-120; Pharmacia Fine Chemicals, Inc., Piscataway, N. J. 08854) was equilibrated with tris(hydroxymethyl)aminomethane HCl (0.1 mol/liter, pH 9.0) containing NaCl (0.1 mol/liter) and subsequently degassed by submitting the gel to reduced pressure created with an aspirator. It was packed into a column (1.3 x 5.0 cm), which was connected in series to a gradient-elution apparatus. The initial volume of starting buffer in the mixing reservoir was 50 ml.

Sepharose G-100-120 (Pharmacia Fine Chemicals) was prepared in tris(hydroxymethyl)aminomethane HCl (0.1 mol/liter, pH 8.0) containing NaCl (0.1 mol/
liter) according to manufacturer’s specifications. Sephadex G-10 (Pharmacia Fine Chemicals) was similarly prepared except that the equilibrating solvent was iron-free water.

Mercaptoacetic acid (thioglycolic acid), 98% pure, was purchased from Matheson, Coleman and Bell, Norwood, Ohio 45212.

Trichloroacetic acid (Mallinckrodt Chemical Works, St. Louis, Mo. 63160) was glass-distilled to remove traces of iron. A 300 g/liter solution was then made with iron-free water.

Sodium acetate (99%+, Grade I, crystalline trihydrate; Sigma Chemical Co., St. Louis, Mo. 63178) was used without further purification to prepare a 4.3 mol/liter solution. It is advisable to test every new batch of sodium acetate, because some batches are intolerably contaminated with iron.

Color reagent: Disodium 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (“ferrozine”; Hach Chemical Co., Ames, Iowa 50010), 80 mg, was dissolved in iron-free water and diluted to 100 ml.

Stock iron standard was prepared by dissolving 1.0 g of pure iron wire (Mallinckrodt) in 15 ml of concentrated HCl. This was diluted to 1 liter with iron-free water. One milliliter of this (equivalent to 1 mg of iron) was transferred quantitatively to a 500-ml volumetric flask, which was then filled to the mark with iron-free water to give a working standard with an iron concentration of 2 µg/ml.

Iron-free water: Purified de-ionized water obtained from a Continental Ultra Pure Water System (Continental Ultra Pure Water Corp., Farmington, Mich. 48024) was iron free.

Human methemoglobin (Type IV, 2X crystallized; Sigma Chemical Co.) was used without further purification. The absorption spectrum of the product in phosphate buffer (0.1 mol/liter, pH 7.0) containing 100 mg of KCN per liter showed maxima at 280, 360, 422, and 540 nm.

Methemalbumin was prepared as follows: 60.5 mg of hemin chloride (Eastman Organic Chemicals, Rochester, N. Y. 14650), dissolved in 32.0 ml of Na2HPO4 buffer (0.1 mol/liter, pH 9.5) containing 100 mg of KCN per liter, was added dropwise to a solution of 400 mg of human albumin (Cohn Fraction V; Sigma Chemical Co.) in 160 ml of water. The entire operation was performed in the cold (0–4°C) and away from direct light, according to previously reported procedures (16, 17). To remove the last traces of unreacted heme remaining after dialysis of the reaction product, 370 mg of the lyophilized methemalbumin was dissolved in 10.0 ml of cold (0–4°C) distilled water and applied to a column (2.2 × 38 cm) of Sephadex G-10 that had been pre-equilibrated overnight with iron-free water. The effluent was collected in 5-ml fractions at a flow rate of 36 ml/h for a period of 3 h. The unreacted heme remained on the upper third of the column. Fractions containing methemalbumin were pooled and lyophilized. The absorption spectrum of the product (methemalbumin) in the pH 7 phosphate buffer showed maxima at 280, 360, and 405 nm. The absorption at 360 nm appeared as a shoulder, probably as a result of masking by the Soret band. The ratio of iron to albumin in this preparation was equimolar.

Rabbit anti-human hemopexin, fluorescein conjugated, was obtained from Behring Diagnostics, Somerville, N. J. 08876.

Procedures

The details of the procedure eventually adopted for the clinical determination of methemalbumin are as follows:

1. Draw blood into Vacutainers (Becton-Dickinson, Rutherford, N. J. 07070) and remove serum as soon as possible.

2. Apply 1 ml of serum to a 1.3 × 5 cm column containing DEAE Sephadex A-50.

3. Elute the column with 35 ml of the pH 9.0 tris(hydroxymethyl)aminomethane HCl buffer containing NaCl (0.1 mol/liter), and discard the eluate.

4. Begin a linear concentration gradient between 50 ml of this buffer and one that is the same except for NaCl concentration (0.6 mol/liter). Maintain the volume in the mixing reservoir at the flow rate of the column.

5. Collect 10 2.5-ml fractions at about 1.5 ml/min, and measure their absorbance (A) vs. a buffer blank at 280 nm, to determine protein concentrations (the molar absorptivity of albumin is 3.48 × 104). Steps 2 through 5 are done at room temperature.

6. Obtain an A405 reading for the peak protein tube as well as the subsequent tube. If the A290/A405 ratio exceeds 30, no methemalbumin is present in the initial sample (Table 2) and no further work is required. If the ratio is less than 30, methemalbumin may be present in the original specimen. In this case, pool the contents of the two tubes and submit them to iron analysis, to distinguish methemalbumin from potential interferences, as follows.

7. Mix 2 ml of the pooled eluate, 3.0 ml of HCl (0.5 mol/liter), and 0.2 ml of mercaptoacetic acid, and allow the mixture to stand at room temperature for 30 min.

8. Add 1 ml of the trichloroacetic acid reagent, cover the tubes, and, after mixing, incubate the tubes in a hot-water bath (80–85°C) for 30 min.

9. After cooling, centrifuge the tubes for 10 min.

10. Label a blank and a sample tube for each determination. To each of these tubes add 2-ml aliquots of supernate and 0.5 ml of the sodium acetate reagent, followed by 0.5 ml of ferrozine for the sample tube and 0.5 ml of water for the blank. Allow the color to develop for 15 min and measure the absorbance of each sample tube vs. its own blank at 562 nm.

11. Take a reagent blank and standards through steps 7–10.

The overall methodology was checked by assaying normal sera, normal hemolyzed sera, normal sera with added methemalbumin, and normal sera with
added methemoglobin. We investigated the behavior of methemalbumin, methemoglobin, and transferrin on Sephadex G-100 as well as on the DEAE-Sephadex. Elution of hemopexin from the DEAE-Sephadex column was monitored by immunodiffusion vs. antihuman hemopexin on an Ouchterlony plate.

Methemalbumin does not appear in blood under normal conditions. Hemoglobin released during hemolysis has a high affinity for haptoglobin, and only after the capacity of haptoglobin to bind hemoglobin in blood is exceeded will free hemoglobin appear in serum. This is demonstrated graphically in Figure 1, in which increasing amounts of hemoglobin have been added to normal serum and eluted from a Sephadex G-100 column. Small amounts of hemoglobin appear in the void volume (about 19 ml) as hemoglobin/haptoglobin complex, and only after the haptoglobin binding capacity has been exceeded does the free hemoglobin appear in the 64,000 molecular weight range (at about 30 ml of total eluate). The peak at 38 ml corresponds to a molecular weight of about 32,000 and represents a free hemoglobin dimer, which results from application of the corresponding tetramer to Sephadex (18). The tetramer and dimer are normally in equilibrium when hemoglobin is placed in solution, with the tetrameric form greatly favored, but the hemoglobin filters down the gel column and as existing dimer is left behind, more and more tetramer dissociates, eventually giving rise to a sizeable concentration of dimer.

In the event that proteinases have been released and allowed to come in contact with hemoglobin (4) (e.g., in hemorrhagic pancreatitis) some of the heme is released from its protein component. The iron of such a heme moiety is oxidized from Fe$^{2+}$ to Fe$^{3+}$ and the resulting heme then associates with either hemopexin, a globulin, or with albumin to form methemalbumin. In patients with intravascular hemolysis, hemopexin is depleted rapidly and most of the heme is bound by albumin (19).

The normal catabolism of hemoglobin in the reticuloendothelial system involves scission of the porphyrin ring to give choleglobin (also called verdo-hemoglobin; a biliverdin/iron/protein complex), which successively undergoes removal of the globin, removal of the iron, and, finally, reduction to yield bilirubin. Bilirubin is transported from extrahepatic reticuloendothelial cells to the liver in combination with albumin. In the event of massive hemolysis, bilirubin concentrations in blood can be expected to exceed normal.

Thus, in any spectrophotometric technique for measurement of methemalbumin, one must consider the following potential sources of interference: free hemoglobin, hemoglobin/haptoglobin complex, hemopexin, bilirubin, and—if iron measurements are involved in the assay system—other iron-containing compounds, such as transferrin.

After considerable experimentation, we decided that ion-exchange chromatography was a good way to separate methemalbumin (isoelectric point, 4.7) from methemoglobin (isoelectric point, 6.8) and transferrin (isoelectric point, 5.9). Figure 2 shows the separation of a mixture of 2.5 mg each of methemalbumin and methemoglobin applied to a DEAE-Sephadex A-50 column (1.3 x 13 cm). Methemoglobin is recovered first (10–25 ml of eluate), with methemalbumin eluting after initiation of the gradient, at a total elution volume of 45–52 ml. Analytical recovery, estimated from addition of known amounts of methemalbumin to normal serum, was about 99%. Separation of the hemoglobin/haptoglobin complex from methemalbumin on DEAE-Sephadex is more difficult and requires rigid pH control, but at pH 9 the separation is virtually complete (Figure 3). Transfer-
rin and hemopexin are eluted from this system under the first protein peak (at about 10 ml). The elution characteristics of transferrin were demonstrated by applying the protein to an appropriate column, those of hemopexin by immunodiffusion vs. fluorescein-conjugated anti-human hemopexin. Thus, elution of serum proteins from a DEAE-Sephadex column with a concentration gradient at pH 9 results in clean separations of methemalbumin from all other iron-containing compounds that may be anticipated in serum derived from an individual with hemolytic disease (Figure 4).

There are also non-iron-containing compounds that absorb in the region 400–425 nm where heme-containing compounds absorb maximally. Bilirubin is one such compound; its absorption band is broad (350–520 nm). If the $A_{280}/A_{405}$ ratio of the peak albumin-containing tube(s) of a serum applied to DEAE-Sephadex and eluted as described is less than 30, the $A_{405}$ contribution originates either from methemalbumin or from a non-iron-containing compound. For such samples, then, an iron determination must be done.

Determination of microgram quantities of iron is inherently difficult because many of the reagents, including water, tend to contain the metal. Furthermore, copper can interfere. For instance, erratic results in our laboratory in the determination of iron in eluates from normal sera were traced to a copper rack used to support the test tubes in the $85^\circ$C water bath for step 8 of the procedure. The quantitative release of iron from heme requires fairly drastic conditions: an acid pH (obtained in this case with the 0.5 mol/liter HCl), heat (30 min heating at 80–85 °C), and the presence of a strong reducing agent. Table 1 gives our recovery figures for iron from methemoglobin. The method is acceptable to absolute values of 0.17 μg of iron; for amounts less than this, results are apt to be erratic.

We measured the analytical recovery of methemalbumin iron taken through the whole procedure, including the ion-exchange chromatography, after adding 1.45, 2.90, or 4.35 μg of iron (as methemalbumin) to 1 ml of normal serum. Recoveries ranged from 98.5 to 102%.

Table 2 shows our results for sera, both normal and abnormal, taken through the entire assay, including

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**Table 1. Analytical Recovery of Iron from Methemoglobin**

<table>
<thead>
<tr>
<th>Vol of stock added, ml</th>
<th>Fe added (μg)</th>
<th>Fe found (μg)</th>
<th>Recovery, %</th>
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<tr>
<td>1.0</td>
<td>3.40</td>
<td>3.36</td>
<td>98</td>
</tr>
<tr>
<td>0.5</td>
<td>1.74</td>
<td>1.71</td>
<td>100</td>
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<tr>
<td>0.2</td>
<td>0.68</td>
<td>0.70</td>
<td>102</td>
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<tr>
<td>0.1</td>
<td>0.34</td>
<td>0.40</td>
<td>117</td>
</tr>
<tr>
<td>0.05</td>
<td>0.17</td>
<td>0.16</td>
<td>92</td>
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*Concentration, 1.0 mg of methemoglobin per milliliter.
Table 2. Methemalbumin Concentrations in Some Sera

<table>
<thead>
<tr>
<th>Number</th>
<th>Sex</th>
<th>Condition</th>
<th>$A_{280}/A_{405}$</th>
<th>$\mu g$ Fe</th>
<th>albumin</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>Na</td>
<td>55.5</td>
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<tr>
<td>2</td>
<td>F</td>
<td>N</td>
<td>51.0</td>
<td>3.7</td>
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<td>3</td>
<td>F</td>
<td>N</td>
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</tr>
<tr>
<td>4</td>
<td>M</td>
<td>N</td>
<td>37.2</td>
<td>0.0</td>
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</tr>
<tr>
<td>5</td>
<td>M</td>
<td>N</td>
<td>34.4</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>N</td>
<td>59.1</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>NH b</td>
<td>50.8</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>NH</td>
<td>24.6</td>
<td>0.0</td>
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</tr>
<tr>
<td>9</td>
<td>F</td>
<td>NH</td>
<td>18.0</td>
<td>11.6</td>
<td></td>
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<tr>
<td>10</td>
<td>F</td>
<td>NH</td>
<td>20.3</td>
<td>1.0</td>
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<tr>
<td>11</td>
<td>M</td>
<td>NH</td>
<td>24.5</td>
<td>9.6</td>
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<tr>
<td>12</td>
<td>M</td>
<td>Acute pancreatitis</td>
<td>22.5</td>
<td>0.0</td>
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<tr>
<td>13</td>
<td>M</td>
<td>Acute pancreatitis</td>
<td>37.3</td>
<td>0.25</td>
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<td>14</td>
<td>M</td>
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<td>8.2</td>
<td>18.6</td>
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<tr>
<td>15</td>
<td>M</td>
<td>Hemorrhagic pancreatitis</td>
<td>3.2</td>
<td>98.5</td>
<td></td>
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<tr>
<td>16</td>
<td>M</td>
<td>Hemorrhagic pancreatitis</td>
<td>(a) assayed</td>
<td>3.68</td>
<td>65.5</td>
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<tr>
<td></td>
<td></td>
<td>6 months later; stored</td>
<td>(b) assayed</td>
<td>3.0</td>
<td>69.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>frozen</td>
<td></td>
<td>4.67</td>
<td>62.5</td>
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<tr>
<td>17</td>
<td>M</td>
<td>Hemorrhagic pancreatitis</td>
<td>Replicate aliquots, same sample</td>
<td>2.51</td>
<td>210</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>2.41</td>
<td>182</td>
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<td></td>
<td>2.56</td>
<td>190</td>
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<td></td>
<td></td>
<td>2.64</td>
<td>210</td>
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</table>

$^a$ N = normal serum
$^b$ NH = normal hemolyzed serum

the iron determination. Because their $A_{280}/A_{405}$ ratios exceed 30, one would not normally perform the iron assays for samples 1 through 7 and 13; we did so to indicate the range for the data on iron that one may anticipate for normal controls: 3.7 ± 1.7 (SEM) $\mu g$ of iron per gram of albumin. The upper limit of the normal range (mean ± 3SD) is thus 20 $\mu g$/g.

Reproducibility (CV) of the method, illustrated with sample 17, is within ±7%. Frozen storage of samples does not affect results, as shown by sample 16; however, this determination is generally an emergency procedure, and so sample storage is unusual.

Serum from patients with acute pancreatitis (samples 12 through 14) gave values ranging from 0 to 18.6 $\mu g$/g. Four different sera (from three patients, samples 15 through 17) presumed to contain methemalbumin on the basis of clinical and histological evidence of hemorrhagic pancreatitis contained 98.5, 65.5, 62.5, and 198 (av) $\mu g$/g. Based on the ratio of 56 g of iron per mole (67 000) of methemalbumin, these values would respectively correspond to 118, 78, 75, and 237 mg of methemalbumin per gram of albumin. (Serum albumin concentrations for these samples were 33, 35, 31, and 30 g/liter, as measured with the Technicon SMA 12/60.) Because analytical recoveries from DEAE-Sephadex for albumin, like those for methemalbumin, range from 98–102%, these data could be reported as 3890, 2730, 2330, and 7110 mg of methemalbumin per liter, respectively, if desired.

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References