Use of Ion-Exchange Resin in Preparing Erythrocytes for Magnesium Determinations

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A rapid, accurate method is described for determining magnesium in red blood cells. Dowex 50 resin was used to separate magnesium from calcium and other cellular constituents. Magnesium in the eluate was titrated with EDTA, with Erichrome Black T as indicator; the end point was determined spectrophotometrically. Magnesium in the erythrocytes of 17 healthy adults averaged 4.57 ± 0.16 (SD) mEq/liter of packed cells, with a range of 4.32 to 4.88, narrower than reported before in the literature.

Evidence has accumulated during the past 10 years to indicate that disturbances in magnesium metabolism can coexist with a variety of unrelated clinical disorders (1, 2). Estimation of magnesium has become a routine laboratory procedure in many hospitals. As a result, it has become apparent that serum values do not always substantiate a clinical diagnosis of abnormal magnesium metabolism, and that magnesium deficiency may be present in spite of normal serum values. The estimation of intracellular magnesium may help to establish an accurate diagnosis in these cases. Usually a sample of skeletal muscle is chosen to represent tissue cells generally. Muscle biopsy is not a routine laboratory procedure, but erythrocytes are readily available.

It is not established unequivocally that erythrocyte magnesium concentration directly parallels the intracellular magnesium concentration of other tissues in conditions of abnormal magnesium metabolism. However, mounting evidence indicates that red blood cell magnesium is a more accurate guide to diagnosis and treatment than serum values alone. This has been so in conditions such as uremia, delirium tremens (3), and other disorders (4, 5).

A simple, reliable method for determining red blood cell magnesium, therefore, would be valuable in cases in which the clinical diagnosis differs from the serum findings. This paper describes a method in which a cation-exchange resin was used to separate magnesium from calcium and other cellular constituents; the magnesium was subsequently titrated with EDTA and the end point was detected spectrophotometrically with Erichrome Black T as the indicator.

The ion-exchange procedure that was developed may be described briefly as follows: 6N HCl removed iron and other heavy metals from the resin; the exchanger in the K+ form was the most efficient for cation separation; 0.16M NaCl washed protein and anions through the columns; the mixed KCl + NaCl solution used for elution completely separated magnesium from calcium, as shown by spot tests of the eluate fractions.

Methods and Materials

Triply distilled water was used to prepare all solutions, which were stored in polyethylene containers. Water from a Barnstead still was redistilled twice in a Corning, all-Pyrex distilling apparatus. All apparatus was acid-washed, rinsed, and then washed with detergent. Exhaustive rinsing, first with tap water, then with distilled water, and finally with triply distilled water, completed the treatment. All salts used were reagent grade.

Preparation of Cation Exchanger and Tests of Operation

Magnesium standard. A standard containing 2 mEq of magnesium ion per liter was made from a stock solution containing 20 mEq/liter. The stock solution was prepared from magnesium metal dissolved in redistilled 6N HCl, and made to volume with triply distilled water.

Spot test for magnesium. Thirty-five milligrams of Erichrome Black T (Baker, AR) was dissolved in 100 ml of absolute alcohol. This was prepared freshly at least once a month and was added to an ammonium chloride buffer before use in the spot test.

To make the ammonium chloride buffer (pH 10), 7.5 g of ammonium chloride in 57 ml of concentrated redistilled NH₄OH (14M), was diluted to 100 ml with triply distilled water.

The dye and buffer were mixed just before use in proportions of 1 volume of dye solution to 2 volumes of buffer; the solution should be used within 30 min of mixing. To detect magnesium, 2 drops of dye solution were added to 6 drops of eluate from

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each test tube and placed in a porcelain spot plate. On addition of the dye, the color in positive fractions ranged from pink, in those containing most magnesium, to purple-violet, in fractions containing a trace only. All of these colors are easily distinguished from the clear blue color of the negative fractions.

**Spot test for calcium.** Glyoxyal-bis(2-hydroxyanil) (GBHA), 0.4 g, was dissolved in 100 ml of absolute alcohol and added to an ammonium hydroxide solution before use in the spot test. Five grams of NaOH was dissolved in 100 ml of triply distilled water. Two milliliters of GBHA solution was mixed with 0.3 ml of NaOH (5 g/100 ml) and used within 5 min of mixing. The procedure resembled that for magnesium except that the alkaline GBHA solution was substituted for Eriochrome Black T. A strongly positive test gives a red color and a completely negative test a yellow color. Intermediate concentrations of calcium produce an orange color.

**Salt solutions for operating ion-exchange columns.** KCl, 3M, was used for charging the resin, and 0.16M NaCl for equilibration and hemoglobin elution. For eluting magnesium from the column a mixed salt solution was used which was 1M with respect to KCl and 0.5M with respect to NaCl.

**Preparation of the cation-exchange resin.** All procedures involving the use of the resin were critically tested by using plasma or solutions of calcium and magnesium salts; details of these tests will not be presented here. The resin (Dowex 50W×12, 200–400 mesh, Baker AR) was prepared in bulk as follows:

**Step A.** To ensure that all of the resin was initially in the H⁺ form, approximately 150 g of Dowex (sufficient for 30 columns) was placed in a 1-liter Erlenmeyer flask and 500 ml of 6N HCl added; it was allowed to stand for 30 min with frequent stirring; the supernatant fluid was then decanted. The process was repeated twice with fresh 6N HCl. This procedure also removed any iron which was present as a contaminant (see below). The resin was then washed free of acid with successive 500-ml volumes of distilled water until the pH of the supernatant wash liquid was neutral.

**Step B.** Preliminary experiments showed that the sharpest separation of cations present in serum and erythrocytes occurred when the resin was in the K⁺ form. The resin was converted from the H⁺ to the K⁺ form by adding successive 500-ml volumes of 3M KCl to the slurry, and stirring vigorously with each addition. Thirty minutes was allowed to elapse after stirring, and substitution of K⁺ ion for the H⁺ ion was considered complete when the pH of two successive supernatants was neutral, which required five or six 500-ml volumes of KCl solution. The resin was then washed with several volumes of triply distilled water until the supernatant liquid ceased to give a positive test for chloride with a silver nitrate solution.

**Step C.** The resin was then dried overnight at 100°C, cooled, and stored in a tightly stoppered polyethylene container.

**Preparation of chromatography columns.** Columns of resin 5.5 cm in length were contained in 10-ml pinchcock burets with i.d. of 0.8 cm. A 12-mm piece of soft Nalgene tubing placed over the delivery tip ensured uniform delivery and a means for closing the column. A small plug of chemically clean, coarse glass wool in the base of the buret prevented resin leakage without influencing the rate of flow.

Only 2.5 g of the dry resin was required to form a 5.5 × 0.8-cm column when wet. Thus, depending on the number of columns required, a quantity of resin was weighed, placed in a beaker to which five resin volumes of 0.16M NaCl was added to equilibrate it (for at least 30 min) in preparation for hemoglobin removal. Excess NaCl was then removed, and the slurry of resin poured into the burets and packed under air pressure to a height of 5.5 cm. Further NaCl solution equivalent to 6 bed-volumes (approximately 18 ml) was run through each column, which was then closed and ready for use.

The process described above was carried out on all newly purchased resin. After one chromatographic run, the used resin could be collected and treated in a way similar to that already described and was thus made ready for reuse.

**Separation and detection of iron.** It has been established that the average iron content of human erythrocytes is 104 mg/100 g of red cells (6). Although it may be assumed that most of the iron present is eluted still attached to the hemoglobin molecule (Connell, G. E., personal communication), it was necessary to establish that any iron which did exchange with the resin was not eluted with the magnesium, as the presence of such heavy metal interferes with subsequent magnesium determination. Accordingly, spot tests were carried out for iron as follows: 2 ml of a 1-in-8 dilution of red cells was run through a freshly prepared resin column and eluted with the mixed solution of KCl and NaCl (total 1.5M). Twelve 5-ml fractions were collected into polyethylene tubes, each containing a few crystals of solid sodium thiocyanate and three drops of 6N HCl. Each fraction gave a completely negative result, as indicated by the absence of the yellow-brown discoloration around the crystals that is always seen when iron is present. Hence, the mixed salt solution obviously did not elute iron from the column. The presence of some iron in the resin was shown when 50 ml of 6N HCl was put through the column after the salt treatment. Five-milliliter fractions were collected and iron, detected by the presence of the yellow-brown discoloration, was present in fractions 3 to 6 (10 to 30 ml) of this eluate. This procedure proved satisfactorily that
no iron was eluted with the mixed-salt resin used for magnesium elution, but demonstrated the need for the iron-removal process described above.

Elution of magnesium. Numerous systems of salt solutions had previously been studied for use in isolating and recovering magnesium in plasma.

In these experiments with plasma, we found that magnesium was separated in the smallest volume, and completely from other plasma constituents including calcium, when (a) the resin was initially in $K^+$ form, (b) protein and anions were washed through with 0.16M NaCl, and (c) magnesium was eluted with a mixed 1M KCl and 0.5M NaCl solution at the rate of 0.4–0.5 ml/min. In this system no premature elution of magnesium occurred during the passage of 0.16M NaCl, and magnesium was completely eluted between the 7th and 18th ml.

We decided, therefore, to assess this system for use in the elution of erythrocyte magnesium. Preliminary studies on standard solutions of calcium and magnesium salts and dilute erythrocytes were carried out with the above system. Two-milliliter aliquots of eluate were collected. Fractions containing magnesium were detected by the spot test already described. Repeated test runs with both the standard solutions and dilute erythrocytes showed magnesium to be present in the 4th to the 14th ml, with maximum elution in the 6th to 10th ml. Although the spot test described is sensitive enough to detect 0.025 μEq of magnesium, it was found during studies on the separation of calcium and magnesium in serum that minute, but significant, quantities of magnesium may escape detection. However, by spectrophotometric analysis, magnesium in the standard solution was completely recovered when eluate fractions 1 through 10 (total, 20 ml) were analyzed. Therefore, in the elution of erythrocyte magnesium, the first 20 ml of eluate were routinely collected and all results subsequently presented were obtained on this fraction.

Final Experimental Procedure

Blood Sampling and Preparation of Erythrocytes

Syringes were thinly coated with a minimum of heparin solution (1 g/100 ml of isotonic NaCl) and a fresh, dry needle attached before venipuncture. Two milliliters of venous blood was drawn from the antecubital vein of healthy laboratory personnel or medical students, without tourniquet, and placed in a 12-ml polyethylene tube.

The blood was centrifuged at 3500 rpm for 10 min and the plasma removed with a Pasteur pipet. The erythrocytes were then washed twice with approximately 10 ml of normal saline, centrifuging for 5 min between each wash. The cells and saline were mixed by gently inverting each tube four or five times. The buffy coat (white blood cells) and platelets were poured off with the saline from the first wash. After the second wash, the red cells were transferred to a 15-m1 Pyrex centrifuge tube (heavy-duty) and washed a third time with 10 ml of saline. After centrifuging for 10 min the volume of red cells was observed and recorded. The supernatant saline from the last wash was pipetted off as completely as possible without disturbing the red cells on the surface. The erythrocytes were diluted with seven volumes of water to give the required magnesium concentration, shaken vigorously to produce hemolysis, and thoroughly mixed.

Operation of Column

Two-milliliter samples of the diluted red cells were allowed to trickle down the side of the buret to the surface of the resin from a 2-ml “blow-out” pipet. Material adhering to the sides of the buret was washed into the column with approximately 3 ml of the 0.16M NaCl solution from a polythene bottle fitted with a fine delivery tip. After complete absorption of the cells and saline wash into the resin, the buret was filled with successive volumes of a 0.16M NaCl solution until approximately 40 ml had passed through the column. The resin was then completely freed of hemoglobin. When the last of the 40 ml of saline was entirely absorbed into the resin, magnesium was eluted. A calibrated beaker or a 20-ml volumetric flask was placed under the delivery tip, and the buret filled with the mixed KCl and NaCl (total 1.5M) salt solution. The required rate of elution was achieved by refilling the buret each time 5 ml of eluting fluid had been delivered. When exactly 20 ml of eluate was collected the flow was stopped, and the resin was collected for reprocessing as previously described. The whole process requires about 30 min.

Determination of Erythrocyte Magnesium

Magnesium was measured by the EDTA spectrophotometric titration method of Campbell (7). As the erythrocyte magnesium is finally estimated in 20 ml of 1.5M salt solution, particular care was taken to obtain an accurate measurement of the magnesium present as a contaminant in the salts. For this reason two blanks were run with each batch of red cells—i.e., 20 ml of the mixed salt solution was poured through a freshly prepared column and collected as previously described. Magnesium was estimated in the eluted salt solution and the mean value obtained on these blanks was subtracted from the final EDTA titration reading obtained on the erythrocyte samples.
Results

Recovery of Magnesium

The following experiments showed that there was no loss of magnesium from the red cells during cell washing and hemoglobin elution and also that no loss could be attributed to incomplete magnesium elution.

Cell washing. In six samples of red cells the supernatant saline from each of the three washes was collected and spot-tested for magnesium and calcium. Spot tests showed both calcium and magnesium in the first wash, very little of either in the second, and none in the third. The magnesium in the first wash presumably represented that present in the plasma adhering to the cells after their separation from plasma. If it had come from leaking red cells, the magnesium concentration would have been greater in the supernatant liquid of subsequent washings, not less, as was actually found.

In addition, separate calcium and magnesium estimations were performed on the last wash from each red blood cell sample. The volumes of EDTA required for these titrations were exactly the same as those required for the titration of an equivalent volume of 0.16M NaCl. It should be pointed out that the estimation of calcium was necessary to ensure that the supernatant liquid was completely free of this cation, since EDTA complexes with both calcium and magnesium at the pH of the magnesium titration.

These findings suggest that the red cells did not lose magnesium during the washing procedure.

Premature and incomplete elution. It was necessary to know that magnesium was held by the resin during the initial passage of the 0.16M NaCl solution, which removed the hemoglobin and anions from the red cell hemolysate. Previous experience with serum had shown that magnesium was not eluted with 0.16M NaCl, but the higher concentration of magnesium in the erythrocyte made further experiments necessary: the volume of saline (2 × 20 ml) used for hemoglobin elution was run through and collected from each of four columns, and the magnesium estimated after the hemoglobin had been precipitated with trichloroacetic acid (10 g/100 ml). Again the titers (in ml of EDTA) were identical to those of the blank.

As mentioned above, 20 ml of the mixed KCl and NaCl solution was used to remove magnesium completely from the resin. To test the possibility of incomplete elution a further volume of 25 ml of mixed KCl and NaCl solution was put through each of the six columns after the collection of the initial 20 ml, which was presumed to contain the erythrocyte magnesium. Again, the EDTA titer was identical to that obtained for 25 ml of the mixed-salt solution from two freshly prepared columns, previously equilibrated for 30 min with the solution of mixed salts.

These experiments suggest that neither premature nor incomplete elution of magnesium occurred, and, therefore, that all magnesium originally present in the sample was contained in the first 20 ml of eluate collected and subsequently titrated.

Test of Dilution

The accuracy of the dilution method used was also investigated. Three separate dilutions were made from each erythrocyte sample obtained from the first nine subjects studied. Separate chromatographic runs were made on each dilution, and the eluate was analyzed for magnesium. Results of triplicate determinations are shown in Table 1.

Normal range. The subjects were 17 healthy laboratory personnel and medical students, 19 to 46 years old. Nine were men and eight women.

The mean erythrocyte magnesium concentration was 4.57 mEq/liter of packed red cells, with a range of 4.32 to 4.88, and a standard deviation of ±0.16 mEq/liter. The normal value for each subject represents the average of three chromatographic runs and determinations.

Discussion

The similar tendencies of calcium and magnesium to form insoluble salts and chelates have long plagued analysts. We show here that a cation exchanger is an excellent tool for rapidly separating

| Table 1. Effect of Dilution on Magnesium Concentration in Red Cells from Nine Normal Subjects (Averages of Triplicate Analyses) |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Dilution no.     | Erythrocyte magnesium, mEq/liter of packed cells |                           |                           |                           |                           |                           |                           |
| 1                | 4.72             | 4.96             | 4.48             | 5.04             | 5.12             | 4.64             | 4.40             | 4.40             |
| 2                | 4.64             | 4.64             | 4.48             | 4.64             | 4.72             | 4.64             | 4.32             | 4.40             |
| 3                | 4.56             | 4.40             | 4.80             | 4.96             | 4.64             | 4.56             | 4.80             | 4.48             |
| Mean             | 4.64             | 4.67             | 4.58             | 4.88             | 4.82             | 4.61             | 4.48             | 4.42             | 4.37             |
Table 2. Normal Values for Erythrocyte Magnesium Reported in Literature, a Summary of Analytical Method Used

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>No. subjects</th>
<th>Preparation of sample</th>
<th>Erythrocytes</th>
<th>Method</th>
<th>Erythrocyte magnesium, mEq/liter</th>
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<tr>
<td></td>
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<td>Whole blood*</td>
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<td>Mean ± SD</td>
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<tr>
<td>4</td>
<td>77</td>
<td>Hb pptn.; calcium oxalate pptn. over 72 h</td>
<td>Erythrocytes weighed; Hb pptn.; calcium oxalate pptn.; anal. of supernatant fluid</td>
<td>EDTA</td>
<td>5.30 ± 0.53</td>
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<tr>
<td>8</td>
<td>5</td>
<td>...</td>
<td>Hb pptn.; Fe removed with Cupferron; analy-sis supernatant fluid</td>
<td>Titan yellow</td>
<td>5.25</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>Erythrocytes ground in a 1:5 dilution; Hb and calcium oxalate pptn.; Fe extracted; Mg anal-insupernatant fluid</td>
<td>...</td>
<td>EDTA</td>
<td>5.29 ± 0.34</td>
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<tr>
<td>10</td>
<td>?</td>
<td>1:2 diln. of washed erythrocytes; calcium oxalate pptn.</td>
<td>...</td>
<td>EDTA</td>
<td>4.67 ± 0.92</td>
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<td>11</td>
<td>58</td>
<td>Washed 1:2 dilution; Hb pptn.</td>
<td>...</td>
<td>8-Hydroxy-quinoline</td>
<td>5.5 ± 0.44</td>
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<td>12</td>
<td>Erythrocytes ashed; calcium oxalate pptn.</td>
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<td>Mg amm. PO₄ ppt.</td>
<td>3.35</td>
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<td>Whole blood ashed; calcium oxalate pptn. iron-free supernatant fluid</td>
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<td>Mg amm. PO₄ ppt.</td>
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<td>14</td>
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<td>As for ref. 8</td>
<td>Titan yellow</td>
<td>4.78 ± 0.74</td>
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<tr>
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<td>Erythrocytes ashed</td>
<td>...</td>
<td>8-Hydroxy-quinoline</td>
<td>4.60</td>
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<td>Here</td>
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<td>Isolated of magnesium on ion-exchange resin</td>
<td>EDTA</td>
<td>4.57 ± 0.16</td>
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</table>

* Erythrocyte magnesium is calculated from the hematocrit and analyses of plasma and whole blood.

calcium from the magnesium of the erythrocyte at neutral pH, prior to the analysis for magnesium; ashing is unnecessary. The resin, used as described, also removes hemoglobin and interfering anions and this procedure can precede any method chosen for the final determination of magnesium. It is particularly suitable for complexometric procedures, currently used when absorption spectrophotometry is not available.

Data from the literature are presented in Table 2 along with an indication of the procedures used. The range of values we found for normal subjects suggests a much more precise magnesium homeostasis in cells than has hitherto been observed.

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References