Direct Spectrophotometry of Magnesium in Serum after Reaction with Hexokinase and Glucose-6-phosphate Dehydrogenase

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We describe a simple method for determining magnesium in serum by using hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The method is based on determination of the reaction rate of hexokinase activated by Mg2+, which participates in the hexokinase reaction as the substrate in the form of a Mg·ATP2− complex. The reaction rate is determined from the change in absorbance at 340 nm as NADPH is produced by the action of glucose-6-phosphate dehydrogenase. This simple and rapid spectrophotometric method does not require expensive instrumentation, but results correlate satisfactorily with those obtained by atomic absorption spectroscopy. Thus, the present method gives a "true" value for magnesium in serum, a value appreciably lower than that obtained by an earlier colorimetric method, the Xyliol Blue II method (Biochem Med 7: 208–217, 1973), which lacks specificity.

The significance of magnesium concentrations in serum has been better appreciated in recent years. Total magnesium is most accurately and precisely determined by atomic absorption spectrophotometry (1, 2), but this requires expensive instrumentation. A widely used colorimetric method involves Xyliol Blue II (3, 4), which undergoes a chelating reaction with magnesium and therefore is not strictly specific for magnesium. To improve the specificity while keeping the instrumentation suitable for a routine clinical laboratory, we developed an entirely new approach by using magnesium-dependent enzymes: hexokinase (HK; EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49). Among the many enzymatic methods proposed for clinical analyses, almost all have been devised for the selective analysis of nonprotein organic constituents of serum. Except for phosphorus assays (5, 6), the present assay is one of the few for inorganic constituents.

The proposed enzymatic method for magnesium in serum is based on the following sequence of reactions:

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\text{d-glucose} + \text{Mg} \cdot \text{ATP}^{2-} \xrightarrow{\text{HK}} \text{d-glucose-6-phosphate} + \text{Mg} \cdot \text{ADP}^{2-} \\
\text{d-glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{d-gluconolactone-6-phosphate} + \text{NADH} + \text{H}^+ 
\]

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(1) \\
(2)
\]

Mg2+ is generally regarded as the activator of HK, but it is actually involved in the HK-catalyzed reaction in the form of a substrate, i.e., Mg·ATP2− complex. Its concentration can be determined by measuring the reaction rate of HK in terms of the increase of absorbance at 340 nm for NADPH production in the second reaction. Under appropriate conditions, the overall rate should be proportional to the amount of the activated HK, which in turn should be proportional to the amount of Mg2+ present.

Materials and Methods

Apparatus. To measure absorbance, we used a Hitachi (Tokyo, Japan) Model 320 spectrophotometer with a cell holder which was kept at constant temperature, or a Shimadzu (Kyoto, Japan) Model UV-120-01 spectrophotometer.

Materials. Both enzymes, HK and G6PDH (140 kU/g; Boehringer-Mannheim-Yamanouchi, Tokyo, Japan) were prepared from yeast. NADP+ and ATP were from Kohjin Co., Tokyo, Japan. A standard solution of magnesium (41.1 mmol/L) was obtained from Nakarai Chemicals Co., Kyoto, Japan. All other chemicals were commercially available and of analytical grade.

Procedures. For the manual enzymic assay with HK and G6PDH, pipette into each of three test tubes 3.0 mL of Tris HCl buffer (50 mmol/L, pH 8.5) containing 15 mmol of glucose, 0.5 mmol of NADP+, and 0.5 mmol of ATP per liter (reagent I). Add 20 µL of distilled water into the first tube (reagent blank), 20 µL of the standard magnesium solution (2 mmol/L) into the second tube (standard), and 20 µL of serum into the third tube (test). Pre-incubate at 30 °C for 5 min, then start the reaction by adding 50 µL of enzyme solution—HK 14 kU/L and G6PDH 28 kU/L—(reagent II) to each tube. Incubate for 15 min at 30 °C, then add 0.1 mL of a 200 mmol/L solution of tetra-sodium EDTA to stop the HK reaction. Measure the absorbance of the standards and tests at 340 nm vs the reagent blank.

Reagent I was stable for two weeks, and reagent II for one week, in a refrigerator.

For comparison, we determined magnesium concentrations by atomic absorption spectrophotometry (2). We diluted 0.1 mL of serum or standard magnesium solutions with 5.0 mL of a 2.00 g/L LaCl3 solution before analysis with a Shimadzu Model AA-646 atomic absorption spectrophotometer. We also determined magnesium by the Xyliol Blue II method (4), using an Hitachi Model 726 discrete automatic analyzer.

Results

Optimization Studies

Formation of the Mg·ATP2− complex and the effect of the concentration of ATP. As Figure 1 shows, for a given molarity of ATP, the HK reaction rate increased with the concentration of Mg2+ up to that approximating the concentration of ATP, then tended to level off. Figure 1 also shows that the overall rate at lower Mg2+ concentrations decreased as the concentration of ATP was increased, consistent with the known inhibition of HK by ATP (7). We chose 0.5 mmol of ATP per liter as the optimum concentration for the following experiments.

pH. The pH optima for HK and G6PDH for the respective reactions were at about pH 8.0 and 9.0. Therefore, we used the enzymes in combination in Tris HCl buffer at pH 8.5.

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Activity ratio between HK and G6PDH. The ratio of activities of HK and G6PDH commonly used in determining glucose in serum is 1:1, but this often produces a lag phase before the reactions proceed to linearity. We found that the lag time in determining magnesium in serum could be eliminated if the HK/G6PDH ratio was 1:2; therefore, we use 0.7 U of HK and 1.4 U of G6PDH per assay tube.

Concentration of magnesium. As shown in Figure 2, the HK–G6PDH reactions proceeded to a certain extent even in the absence of exogenously added Mg²⁺ (see "blank"), perhaps because the reagents used contained trace amounts of magnesium, as indeed we verified by atomic absorption spectrometry of the glucose, NADP⁺, ATP, HK, and G6PDH (but not for the Tris) used in this study. We calculated that these amounts of magnesium would total about 84.5 ng per assay tube, corresponding to a magnesium concentration of 0.16 mmol/L, a suitable value for the reagent blank.

Evaluation of Analytical Performance

Enzymic reaction curve and linearity of standard curve: Enzymic reaction curves of the standards and human serum samples in this assay for magnesium (Figure 2) all showed perfect linearity for at least 30 min after the start of the enzymic reaction, with no visible lag phase. Therefore, under the conditions we used, the rate of the reaction could be calculated simply by comparing the absorbance at 340 nm after a 15-min incubation with that for the reagent blank, without making continuous recordings. To stop the reaction before measurement, we added 0.1 mL of the EDTA solution to the incubation mixture at the 15th minute of reaction. Absorbance varied linearly with the concentration of magnesium, up to 3 mmol/L.

Precision and reproducibility: We repeatedly analyzed two specimens of pooled human serum with low and high magnesium concentrations during 10 days. The present method gave satisfactorily precise and reproducible results: for serum containing 0.96 mmol of magnesium per liter, the within-day CV was 2.2%, the day-to-day CV 3.3%; for 2.88 mmol/L, these respective CVs were 1.3% and 1.8%.

Analytical recovery: A sample of pooled human serum of known magnesium concentration was supplemented with magnesium to give final total concentrations of 0.9 to 3 mmol/L. By the present method we could account for 98 to 103% of the added magnesium.

Comparison with other methods: For the comparison studies we used serum samples from a hospital population. Results by the present method compared well with the atomic absorption measurement, \( r = 0.989 \) (Figure 3A), over a wide range of magnesium values. Agreement with results by the Xylidyl Blue II method, obtained with use of the Hitachi 726 analyzer, was less exact: \( r = 0.934 \) (Figure 3B).

Interfering ions: Among the several ions tested (Table 1), only Mn²⁺ showed any substantial positive interference, at 0.54 mmol/L. However, the usual concentration of Mn²⁺ in

Fig. 1. Effect of magnesium concentration on the overall rate of the HK-G6PDH reaction at ATP concentrations of 0.25 (○), 0.50 (●), and 1.50 (△) mmol/L. Final concentrations of both magnesium and ATP are shown. Procedure as described in the text, but without the addition of EDTA.

Fig. 2. Recordings of the reaction rate for the standard magnesium solutions (0.5–3.0 mmol/L) and six human serum samples (a–f), and (inset) a plot for calibration.

The standard curve was obtained as described in the text.
serum is about 18 mmol/L (8), which should not interfere.

Discussion

In the HK-catalyzed reaction, Mg\(^{2+}\) is involved as a part of the substrate Mg·ATP\(^{2-}\) complex. When the concentration of ATP is much higher than that of Mg\(^{2+}\), the amount of the complex formed depends on the concentration of Mg\(^{2+}\), which can thus be determined by measuring the reaction rate of HK. Once the Mg·ATP\(^{2-}\) is converted by the action of HK into Mg·ADP\(^{2-}\), however, Mg\(^{2+}\) tends to dissociate from the latter complex and again form Mg·ATP\(^{2-}\), because the affinity of ATP for Mg\(^{2+}\) is about 10-fold that of ADP (9). As a result, Mg\(^{2+}\) initially present in the reaction mixture will be recycled in the HK reaction, and the amount of the final product NADPH will thus exceed the stoichiometry expected from reactions 1 and 2 above. In fact, the number of moles of NADPH calculated from the increment in the absorption at 340 nm for 15 min in the standard curves is about fivefold the number of moles of magnesium added to the reaction mixture. Such recycling of magnesium is of course advantageous, resulting in higher sensitivity for the present method.

Approximately 35% of magnesium in serum is protein-bound, mainly to albumin, and 65% is present as ionized magnesium, Mg\(^{2+}\). We developed the present method to determine Mg\(^{2+}\), the form of magnesium required by HK. However, we found that the results obtained by the present method coincided very well with those obtained by the atomic absorption measurements, which determine the total magnesium in serum (Figure 3A). Because the affinity of ATP for Mg\(^{2+}\) is far stronger than that of serum proteins, we postulate that the excessive ATP added to the incubation medium sequestered not only free Mg\(^{2+}\) but also the protein-bound magnesium to form the Mg·ATP\(^{2-}\) complex.

The present method is relatively simple, yet gives high accuracy and precise results. Furthermore, it can be applied to automated analyzers. A similar principle for determining inorganic ions through use of specific ion-requiring enzymes should be applicable to analyses for some other inorganic constituents in serum, e.g., calcium and manganese.

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References