Assay of Magnesium in Serum and Urine with Use of Only One Enzyme, Isocitrate Dehydrogenase ($NADP^+$)

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We report a method for assaying magnesium in serum and urine involving only one enzyme, isocitrate dehydrogenase ($NADP^+$)(EC 1.1.1.42), which requires magnesium ion for activity. The enzymatic reduction of $NADP^+$ by isocitrate increases in rate linearly up to at least 20 mmol/L magnesium in the presence of appropriate concentrations of the two metal-chelating reagents, EDTA and glycol ether diamine-*N,N,N′,N′*-tetraacetate. Within-run (n = 20) CVs and day-to-day (n = 10) CVs for sera are ≤1.5% and ≤2.6%, respectively. Analytical recovery of magnesium in sera averages 96–100%. This method is not affected by bilirubin, hemoglobin, or lipemia. The method (*y*) gives the following results correlating with atomic absorption spectrophotometry (*x*): $y = 1.03x + 0.06$ mmol/L (n = 62, $r = 0.995$, $S_{y|x} = 0.03$) for sera, and $y = 1.03x - 0.10$ mmol/L (n = 62, $r = 0.989$, $S_{y|x} = 0.19$) for urines; with the calmagite method (*x*): $y = 0.99x + 0.04$ mmol/L (n = 62, $r = 0.991$, $S_{y|x} = 0.03$) for sera, and $y = 0.98x + 0.03$ mmol/L (n = 62, $r = 0.999$, $S_{y|x} = 0.02$) for urines.

Indexing Terms: *kinetic enzymatic assay/calmagite method,atomic absorption spectrophotometry compared*

Various methods have been used to measure magnesium in biological samples. Atomic absorption spectrophotometry (AAS) (*1*) is an appropriate tool, but the equipment is expensive and difficult to automate, and flammable gases are used.[4] Reported spectrophotometric methods involve xylidyl blue (magon sulfonate or magon) (*2–4*), calmagite (*5, 6*), and methylthymol blue (*7*). The chelating reagents used change color when bound with magnesium at alkaline pH. Although these methods have already been used for assay of magnesium in clinical laboratories, there is appreciable interference by various other cations (*4*) or bilirubin (*8*), or they are unstable when exposed to carbon dioxide in air (*9*). Reported enzymatic methods for assay of magnesium (*10–13*) involve one principal enzyme and one or more auxiliary enzymes. The principal enzymes are activated by ATP–magnesium complex. Those often used are hexokinase (EC 2.7.1.1; HK), glucokinase (EC 2.7.1.2; GluK), and glycerokinase (EC 2.7.1.30; GK). Auxiliary enzymes are glucose-6-phosphate dehydrogenase (EC 1.1.1.49; G6PD) in the methods with HK and GluK, and glycerol-3-phosphate oxidase (EC 1.1.3.21; GPO) and peroxidase (EC 1.11.1.7; POD) in the method with GK.

We report here a novel method that involves only one enzyme, isocitrate dehydrogenase ($NADP^+$) (ICD; EC 1.1.1.42). It catalyzes the following reaction, provided that magnesium is present; thus concentrations of magnesium can be measured by the rates of $NADP^+$ reduction:

$$\text{Isocitrate} + NADP^+ \rightarrow \text{2-oxoglutarate} + NADPH + CO_2$$

Materials and Methods

Apparatus

We used a Model 7150 automated analyzer (Hitachi, Tokyo, Japan) for our magnesium assay. For comparison studies, we used a Model Z-8200 atomic absorption spectrophotometer (Hitachi) and Paramax 720ZX automated analyzer (Dade International, Deerfield, IL).

Reagents

Chemicals. ICD from yeast (10 kU/g at 25 °C) and $NADP^+$ were commercial products of Oriental Yeast, Tokyo, Japan. Monopotassium isocitrate was purchased from Kyowa Hakko, Tokyo, Japan; intrafat 100 g/L solution was from Nippon Pharmaceutical, Tokyo, Japan; bilirubin and hemoglobin (Interference Check A) were from International Reagents, Kobe, Japan; EDTA and glycol ether diamine-*N,N,-N′,N′*-tetraacetic acid (GEDTA) were from Doujindo Labs, Kumamoto, Japan; Tris and ascorbic acid were from Wako Pure Chemical, Osaka, Japan. Magnesium calibrator solution (41.1 mmol/L $MgCl_2$ for AAS) was purchased from Nacalai Tesque, Kyoto, Japan. The calibrator solution (1 mmol/L) and the working solutions of magnesium were prepared by diluting the magnesium calibrator solution (above) with deionized water before use.

Standard reaction mixtures for assay of magnesium. The two reagent mixtures (reagent 1 and 2) were used in a standard procedure for assay of magnesium. Reagent 1 contained, per liter, 5 mmol of isocitrate, 10 mmol of EDTA, and 4 mmol of GEDTA in 100 mmol/L Tris-HCl buffer (pH 9.0 at 25 °C). Reagent 2 contained 10 mmol of $NADP^+$ and 25 kU of ICD per liter of 10 mmol/L potassium phosphate buffer (pH 7.0 at 25 °C). Reagents 1 and 2 were stable for >2 weeks at 4 °C.

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[4] Nonstandard abbreviations: AAS, atomic absorption spectrophotometry; GEDTA, glycol ether diamine-*N,N,N′,N′*-tetraacetic acid; GK, glycerokinase; GluK, glucokinase; GPO, glycerol-3-phosphate oxidase; G6PD, glucose-6-phosphate dehydrogenase; HK, hexokinase; POD, peroxidase; and ICD, isocitrate dehydrogenase ($NADP^+$).
Received May 12, 1995; accepted June 8, 1995.

Assay Procedure

To the Hitachi automated analyzer we added 5 μL of the calibrator solution (1 mmol/L magnesium) or isotonic saline (9 g/L NaCl) for the blank, or the sample (serum or urine specimen), and 240 μL of reagent 1 to the reaction cells, and incubated these for 5 min at 37 °C. To start the enzyme reaction, 60 μL of reagent 2 was added to each. Reaction rates for 3 min between 1 and 4 min (ΔA_{340}/3 min) were also measured after adding reagent 2 at analyzer settings of two-point assay mode. The concentrations of magnesium in samples were estimated on the calibration curve.

Results

Optimization Studies

Effects of concentrations of EDTA on rate. We examined the effects of concentrations of EDTA on reaction rate of ICD. In this experiment, the standard reaction mixtures were used, except that EDTA in reagent 1 and ICD in reagent 2 were varied in concentration without addition of GEDTA in reagent 1, using working solutions of magnesium (0–20 mmol/L $MgCl_2$) for sample. ICD did not show any activity in the absence of $MgCl_2$, whereas the activity increased with increasing concentrations of $MgCl_2$, reaching a maximum at 16 mmol/L with an apparent K_m of 50 μmol/L (Fig. 1). When 10 and 20 mmol/L EDTA were added to reagent 1, the activities were significantly depressed, linearly increasing up to 20 mmol/L $MgCl_2$. We therefore chose to add 10 mmol/L EDTA to reagent 1.

Effects of concentrations of $NADP^+$ and isocitrate on rate. We examined the effects of concentrations of isocitrate and $NADP^+$ on reaction rate of ICD. In this experiment, the standard reaction mixtures were used, except that GEDTA was not added to reagent 1, and isocitrate in reagent 1 and $NADP^+$ in reagent 2 were varied in concentration, using a working solution of magnesium (20 mmol/L $MgCl_2$) for sample. With increasing concentrations of isocitrate, the reaction rate of ICD increased, reaching a maximum at 5 mmol/L isocitrate. With increasing concentrations of $NADP^+$, the reaction rate increased, reaching a maximum at 10 mmol/L $NADP^+$. We therefore chose as the concentrations for getting maximum reaction rate 5 mmol/L isocitrate in reagent 1 and 10 mmol/L $NADP^+$ in reagent 2.

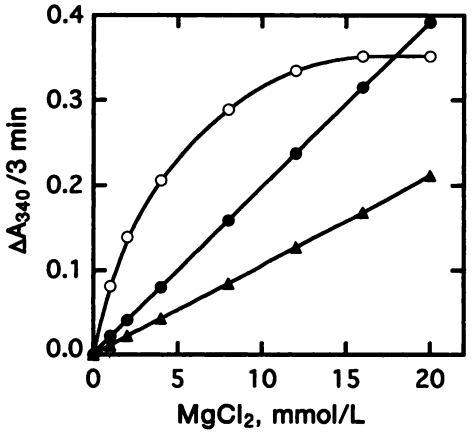

Fig. 1. Effect of $MgCl_2$ concentrations on reaction rate of ICD.

Experiments were carried out with the standard procedure for assay of magnesium, except for the following: GEDTA was omitted from reagent 1; various concentrations of working solutions of magnesium ($MgCl_2$) were substituted for sample. The concentrations of ICD in reagent 2 and EDTA in reagent 1 were, respectively, (○) 50 U/L and 0, (●) 25 kU/L and 10 mmol/L, and (▲) 25 kU/L and 20 mmol/L.

Effects of concentrations of GEDTA on interference of calcium in assay of magnesium. GEDTA is known to be a potent chelating reagent, especially effective against calcium (*14, 15*). We examined the effects of concentrations of GEDTA on the interference of calcium in our assay of magnesium. In this experiment, the standard reaction mixtures were used, except that GEDTA in reagent 1 was varied in concentration, using pooled normal sera supplemented with various concentrations of $CaCl_2$ (Table 1). Assay of magnesium was interfered with by $CaCl_2$ without GEDTA. The interference was neutralized when GEDTA was added to reagent 1; 4 mmol/L GEDTA was sufficient even in the presence of 10 mmol/L $CaCl_2$. We chose to add 4 mmol/L GEDTA to reagent 1.

Effects of pH on linearity of calibration curve for magnesium determination. We examined the effects of pH on the linearity of the calibration curve. In this experiment, the standard reaction mixtures were used, except that 100 mmol/L Tris-HCl buffer and ICD were varied in pH value (25 °C) in reagent 1 and in concentration in reagent 2, respectively, using working solutions of magnesium (0–20 mmol/L $MgCl_2$) for sample. Concentrations of the enzyme were adjusted to give similar reaction rates based on the data of pH–activity profile (data not shown) of ICD in 100 mmol/L Tris-HCl buffer. Of the pH values examined, 9.0 gave the increase of the reaction rate in the most linear manner with increasing concentration of $MgCl_2$ (Fig. 2). We chose to use Tris-HCl buffer, pH 9.0.

Assay Evaluation

Kinetics of reaction and linearity. Fig. 3 shows time courses of reactions carried out by our method for various concentrations of the working solutions of magnesium (0–20 mmol/L $MgCl_2$) as sample. When reagent 2 was added to the mixtures of sample and reagent 1, $NADP^+$ was reduced. Reaction rates of the reduction

Table 1. Effects of GEDTA on interference of calcium in our assay of magnesium.

	GEDTA, mmol/L					
$CaCl_2$, mmol/L	0	2	4	6	8	10
2.5	104[a]	102	102	101	101	101
5.0	106	104	99	97	98	97
10.0	116	106	99	102	99	102

[a]Percunt recovery (100% when no $CaCl_2$ was added).

Concentrations of magnesium were determined by our method, except that the concentration of GEDTA in reagent 1 was varied, using $CaCl_2$-supplemented serum containing 0.87 mmol/L magnesium as sample.

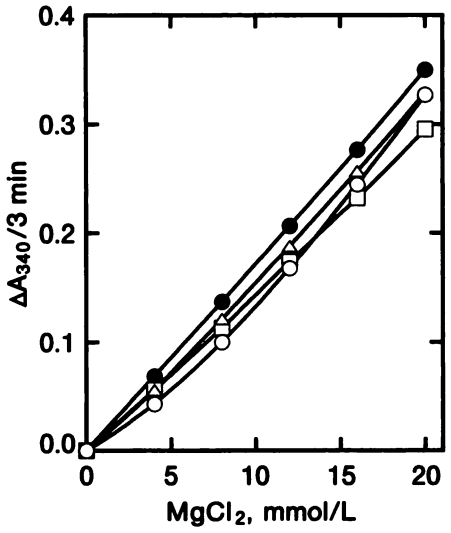

Fig. 2. Effect of pH on linearity of calibration curve.

Experiments were carried out with the standard procedure for assay of magnesium, except that 100 mmol/L Tris-HCl buffer in reagent 1 and ICD in reagent 2 were varied in pH and in concentration, respectively; various concentrations of working solution of $MgCl_2$ were substituted for sample. The ICD concentrations and pH value were, respectively, (○) 3.3 kU/L and 7.5, (△) 5 kU/L and 8.0, (□) 10 kU/L and 8.5, (●) 25 kU/L and 9.0.

after addition of reagent 2 were practically linear for at least 5 min with 0–20 mmol/L $MgCl_2$. We examined the linearity of calibration curve for assay of magnesium in duplicate by our method, using the working solution of magnesium (10 different concentrations: 0–20 mmol/L $MgCl_2$) as sample. The linear relation between reaction rates (ΔA_{340}/3 min) (y) and concentration of $MgCl_2$ (mmol/L) (x) was obtained: $y = 0.0176x + 0.0007$ (r = 1.000, $S_{y|x}$ = 0.0031). The calibration curve for assay of magnesium by our method is straight up to at least 20 mmol/L $MgCl_2$.

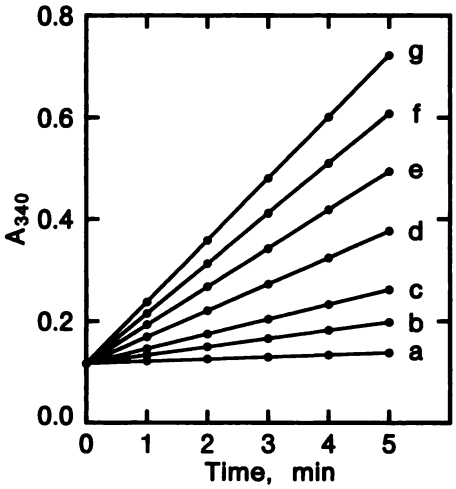

Fig. 3. Time courses for assaying various concentrations of magnesium by our method.

Experiments were carried out with the standard procedure for assay of magnesium, except that various concentrations of working solution of magnesium ($MgCl_2$) were substituted for sample. The concentrations of $MgCl_2$ (mmol/L) were: *a*, 0; *b*, 2.0; *c*, 4.0; *d*, 8.0; *e*, 12.0; *f*, 16.0; *g*, 20.0.

Detection limit. We examined the detection limit of our assay of magnesium, by repeated assay of saline (9 g/L NaCl) as sample: mean = 0.040 mmol/L, SD = 0.015 mmol/L (n = 10). The detection limit, defined as the mean concentration of the magnesium zero sample (saline) + 2.6 SD, was 0.08 mmol/L.

Precision. We examined the precision of our assay of magnesium. The samples used were two pooled human sera, which contained 0.71 and 1.60 mmol/L magnesium, (low and high serum, respectively). Within-run CVs were measured with n = 20, and day-to-day CVs for 10 days (n = 10). The former and latter values were respectively 1.5% and 2.6% with the low serum and 1.3% and 2.4% with the high serum.

Recovery. We examined the recoveries in our assay of magnesium by supplementing the low serum with 0.96, 1.17, and 1.38 mmol/L magnesium, and the high serum with 2.49, 3.48, and 4.55 mmol/L magnesium. Recoveries of the magnesium added were 96–100% with the low serum and 98–100% with the high serum.

Interference. Various substances were examined for their potential effects on our assay of magnesium. The substances to be examined were mixed with nine volumes of a low serum containing 0.82 mmol/L magnesium and then assayed. In some experiments, water was substituted for the substances and used as control sample. The assay was repeated 10 times for each test and control sample. Interference percentage (p) was calculated as follows: $p = [(AM - CM)/CM] \times 100$, where the mean values for the test and control samples are AM and CM, respectively. Substances with $p \leq |3|$ were judged not to interfere. We found that our method was not affected [interference percentages (p) shown in parentheses] by 450 mmol/L NaCl (+1%), 20 mmol/L KCl (0%), 2 mmol/L NH_4Cl (0%), 10 mmol/L $CaCl_2$ (−1%), 0.005 mmol/L $MnCl_2$ (+1%), 0.1 mmol/L $CuSO_4$ (−1%), 0.1 mmol/L $ZnCl_2$ (−1%), 0.1 mmol/L $FeCl_3$ (0%), 90 mmol/L $NaHCO_3$ (−1%), 20 mmol/L Na_2HPO_4 (−1%), 3 mmol/L Na_2SO_4 (+3%), 6.84 mmol/L EDTA (+2%), 556 mmol/L glucose (+1%), 342 μmol/L bilirubin (0%), 30.8 μmol/L hemoglobin (+1%), and 30 g/L intrafat (+1%).

Comparison with another method. We assayed sera from 62 patients and urines from 62 patients independently by AAS, by the Paramax 720ZX automated analyzer calmagite method, and by our method(y) with the Hitachi Model 7150 automated analyzer. The values obtained in comparison with AAS (x) were $y = 1.03x + 0.06$ mmol/L (r = 0.995, $S_{y|x}$ = 0.03) for sera, and $y = 1.03x - 0.10$ mmol/L (r = 0.989, $S_{y|x}$ = 0.19) for urines, and with calmagite method (x) were $y = 0.99x + 0.04$ mmol/L (r = 0.991, $S_{y|x}$ = 0.03) for sera, and $y = 0.98x + 0.03$ mmol/L (r = 0.999, $S_{y|x}$ = 0.02) for urines (Fig. 4). The comparison data of specimens between our method and calmagite method (r = 0.991 for serum and 0.999 for urine) or AAS (r = 0.995 for serum and 0.989 for urine) correlated very well. Results of both methods showed a proportional bias of ≤3% for serum and urine specimens.

Discussion

Various spectrophotometric methods for assay of magnesium in serum involving xylidyl blue (*2–4*), calmagite (*5, 6*), and methyl thymol blue (*7*) as metal-chelating chromogen have been reported. These methods were affected by other cations (*4*) or bilirubin (*8*), and some reagents for assay of magnesium were unstable on setting to automated analyzer (*9*). However, Wills et al. reported that both the calmagite and the automated methyl thymol blue methods could be recommended for magnesium measurements undertaken in clinical laboratories, owing to their acceptable accuracy and precision (*16*). Various enzymatic methods involving one principal and some auxiliary enzymes (*10–13*) have been reported. The principal enzymes used are HK, GluK, or GK, which require the complex of ATP with magnesium as one of the two substrates. The auxiliary enzymes used are G6PD for HK and GluK, and GPO and POD for GK. Since they give linear results only up to 2–3 mmol/L magnesium, the urine used as sample should be diluted >10-fold. In addition, they are interfered with by various cations and metal-chelating reagents such as calcium and EDTA, respectively (*10–13*).

After detailed optimization studies, we developed a method that determines concentrations of magnesium in serum and urine. Characteristic features of our method are: (*a*) it is enzymatic and safe, involving only one enzyme, ICD, which is activated by forming a magnesium–enzyme complex (*17*); (*b*) it contains enough EDTA to give a linear result at ≥20 mmol/L magnesium, making it is effective for assay of urine without dilution; (*c*) it contains GEDTA, so that calcium cannot interfere with the assay; (*d*) it is not affected by bilirubin, hemoglobin, lipemia, or EDTA; (*e*) results of this method correlated well with those determined by the calmagite method and AAS.

Because our enzymatic method is simple and accurate, it may be useful in routine clinical diagnosis with automated analyzers.

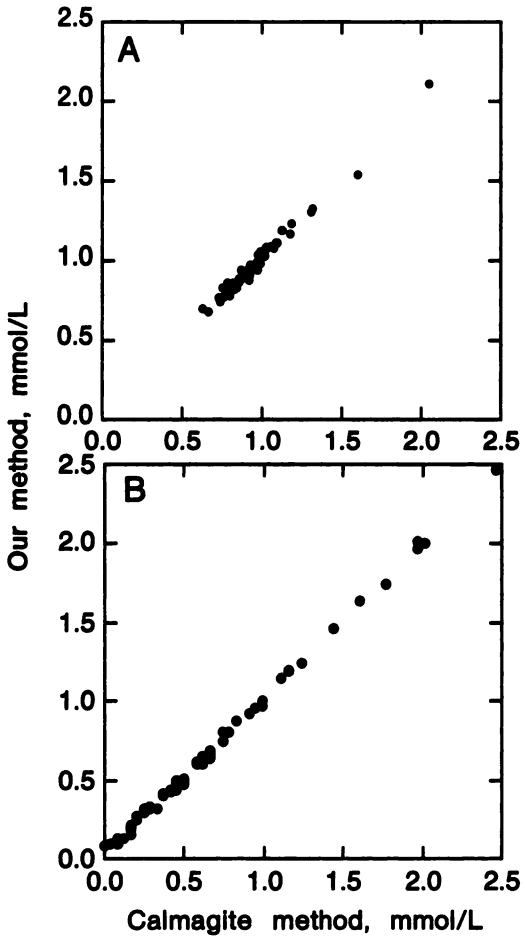

Fig. 4. Correlation between our method and calmagite method for (*A*) sera from 62 patients; (*B*) urines from 62 patients.

We are grateful to T. Horio for discussions and encouragement.

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