# A Kinetik Assay for Determination of Magnesium in biological fluids including EDTA-Plasma

**S. W. Golf, A. Balser*, V. Graef, F. Schneider****

## Zusammenfassung

Die kinetische Bestimmung von Magnesium beruht auf der Reaktivierung von Hexokinase (HK) durch Magnesium. Diese Enzymreaktion wird in Gegenwart von ATP, $NADP^+$, Glukose, Digitonin und anderen Effektoren bei pH 8,5 an die Reaktion der Glukose-6-Phosphat-Dehydrogenase (G-6-P-DH) gekoppelt. Das Verhältnis Probenvolumen zu Reagenzvolumen beträgt 1:200, EDTA-Blut muß zur vollständigen Oxygenierung von Hämoglobin 15 Minuten vorinkubiert werden. Die Meßzeit beträgt 2 Minuten. Anhand einer Standardkurve wird die erhaltene Extinktionsdifferenz bei 334 nm in Magnesium-Konzentration umgerechnet.

Regressionsanalysen der Meßergebnisse, die mit der Atomabsorption bzw. mit der kinetischen Methode aus Plasma oder Vollblut erhalten wurden, ergaben eine Steigung von 1,0, einen y-Achsenabschnitt von 0,1 und einen Korrelationskoeffizienten von 0,92.

Die beschriebenen methodischen Details ermöglichen die Bestimmung von Magnesium in biologischen Flüssigkeiten einschließlich EDTA-haltigem Plasma oder Vollblut. Im Vergleich zu den handelsüblichen enzymatischen Endpunkt-Methoden mit oder ohne Eiweißfällung weist die kinetische Methode folgende Vorteile auf:

a) das Ergebnis ist unabhängig von der Proteinkonzentration in der Probe,

b) Lipämie und Hämolyse beeinflussen das Meßsignal nicht,

c) die Bestimmung von Magnesium in EDTA-haltigen Flüssigkeiten ist möglich,

e) eine Automatisierung der Methode ist möglich.

* This work contains essential parts of the diploma-thesis of *A. Balser*.
Institut für Klinische Chemie und Pathobiochemie, Klinikum der Universität Gießen

## Summary

The kinetic determination of magnesium is based on reactivation of hexokinase (HK) by magnesium and coupling of this enzyme reaction to glucose-6-phospate dehydrogenase (G-6-P-DH) in presence of ATP, $NADP^+$, glucose and digitonin and various effectors at pH 8.5. Sample volume to reagent volume is 1:200, preincubation time for EDTA-blood is 15 minutes in order to complete oxygenation of hemoglobin (Hb). Time of measurement is 2 minutes. The resulting difference of optical density at 334 nm is multiplied by a factor derived from a standard curve in order to obtain magnesium concentration.

Regression analysis of atomic absorption spectroscopy (AAS) vs kinetic method of magnesium concentrations in 120 serum and whole blood samples showed a slope of 1, an intercept of 0.1, and coefficient of correlation of 0.92.

The described methodical details allow the kinetic determination of magnesium concentration in biological fluids, including EDTA containing whole blood and plasma. If compared to an end point method using the reactivation of hexokinase with or without protein precipitation above method displays several advantages:

a) the result is independent of the protein concentration of the sample,

b) lipemic and hemolytic samples exhibit no effect on the result,

c) determination of magnesium concentration in EDTA containing whole blood is possible,

d) an automation of the method is possible.

** Institut für Theoretische Medizin (Physiologische Chemie II), Philips-Universität Marburg

## Résumé

Le dosage cinétique du magnésium repose sur la réactivation de l'hexokinase (HK) par le magnésium et le couplage de cette réaction enzymatique avec la glucose-6-phosphate déshydrogénase (G-6-P-DH) en présence d'ATP, de $NADP^+$, de glucose, de digitonine et de divers effecteurs à pH 8,5. Le rapport volume d'échantillon / volume de réactif est de 1 : 200 et le temps de préincubation du mélange EDTA-sang est de 15 minutes, afin d'assurer une oxygénation complète de l'hémoglobine (Hb). La mesure dure 2 minutes et la concentration de magnésium s'obtient en multipliant la différence résultant de densité optique à 334 nm par un facteur provenant d'une courbe d'étalonnage.

Une analyse de régression effectuée sur les résultats du dosage du magnésium dans 120 échantillons de sérum total par spectroscopie d'absorption atomique (SAA) et méthode cinétique a montré une pente égale à 1, une valeur de 0,1 pour l'ordonnée à l'origine et un coefficient de corrélation de 0,92.

Les détails méthodologiques décrits dans cet article montrent que cette méthode cinétique permet de doser le magnésium dans les liquides biologiques, y compris dans le sang total ou le plasma contenant de l'EDTA. Cette méthode présente certains avantages par rapport à une autre technique impliquant la réactivation de l'hexokinase avec ou sans précipitation protéique:

— le résultat est indépendant de la concentration protéique de l'échantillon,

— les échantillons lipémiques ou hémolytiques n'influencent pas le résultat,

— elle permet le dosage du magnésium dans des échantillons de sang total contenant de l'EDTA,

— elle peut être automatisée.

## Introduction

Magnesium has obtained an intensive clinical attention recently, since it plays a vital role in numerous biochemical events. Among these are the activation of rate-dependent enzymes, its role as the physiologic calcium antagonist, and the regulation of membrane permeability [1].

Determination of magnesium in human body fluids is necessary to evaluate the magnesium status of man. It is not clear, however, in which body fluid magnesium should be determined. Plasma is the obvious choice, since plasma magnesium stays apparently in equilibrium with cellular and extracellular magnesium [2]. Plasma magnesium might, however react to acute stress situations [3], so that it does not necessarily reflect body magnesium. Determination of magnesium in leucocytes is time consuming and not suitable for routine applications.

Magnesium concentration in the erythrocytes reflects the biologically active magnesium within the life span of the red blood cells [4]. It might therefore be regarded, similar to the glycosylated hemoglobin (HbA1c) as an integral value for the fraction of the ion, which was biologically available to the organism.

Determination of magnesium concentration by AAS is considered to be the reference method. Several attempts, however, have been made to establish comparable methods on the basis of chemical reactions or kinetic methods for determination of magnesium concentration in biological fluids, since the equipment for AAS is expensive, and its use is time consuming. Measurement of magnesium in fluids containing chelating reagents, such as EDTA, has not yet been described.

## Materials and methods

### Materials

For the spectrophotometric estimation of magnesium an Eppendorf (Hamburg) photometer 1101 M with a wavelength of 334 nm was used. AAS for determination of magnesium was carried out using an equipment from Evans Electroselenium, Sussex, England.

### Reagents

HK (from yeast) and G-6-P-DH (from yeast) as well as pyridine nucleotides were obtained from Boehringer, Mannheim. The magnesium standard (Titrisol 9949) was purchased from Merck (Darmstadt). All other chemicals were commercially available reagents from Merck, Darmstadt, Serva, Heidelberg, Beckman, München, Behring, Marburg, and Sigma, München. All regents were reasonably free from magnesium. Water for preparing the solutions was purified by ion-chromatography and distillation.

### Control of precision

Determination of magnesium by AAS was characterized by a coefficient of variation (day-to-day) of 1.9 %.

### Statistics

Method comparison was carried out using the procedure from *Passing* and *Bablok* [5]. Calculation of slope and intercept of the standard curve used the least-square-method.

## Results

The optimal conditions for determination of magnesium are listed below (tab. 1):

The procedure for measurement of magnesium includes:

A) 1.0 ml reagent volume, 5 µl sample volume.

B) Temperation for 5 minutes (whole blood 15 min) at 30 °C.

C) Start of the reaction by addition of 20 µl solution containing HK and G-6-P-DH.

D) Determination of change of extinc-tion/min by a readout of 2 min.

E) Calculation of magnesium concentration in the sample on the basis of a standard curve characterized by a linear equation $y = mx + n$. The standard curve was obtained with magnesium concentrations ranging from 0 mmol/l to 2 mmol/l.

Tab. 1: Concentration of reagents for kinetic determination of magnesium in biological samples.

| | |
|---|---|
| Glucose | 15 mmol/l |
| $NADP^+$ | 0.5 mmol/l |
| ATP | 0.5 mmol/l |
| Hexokinase | 14 KU/l |
| Glucose-6-phosphatase | 28 KU/l |
| $CuSO_4$ | 0.1 mmol/l |
| Digitonin | 10 % (w/v) |
| Reagent volume to sample volume 200:1 | |

A typical set of time curves of enzyme activity in dependence on magnesium concentration is shown in fig. 1.

### Standard curve

Fig. 2 shows a typical standard curve for determination of magnesium: the slope is 0.017 (E x l/mmol x min), the y-axis-intercept at an extinction difference of 0.023/min.

### Magnesium content in the water

The magnesium-content in water obtained from various sources ranged from 0.12 mmol/l (purified water dilu-

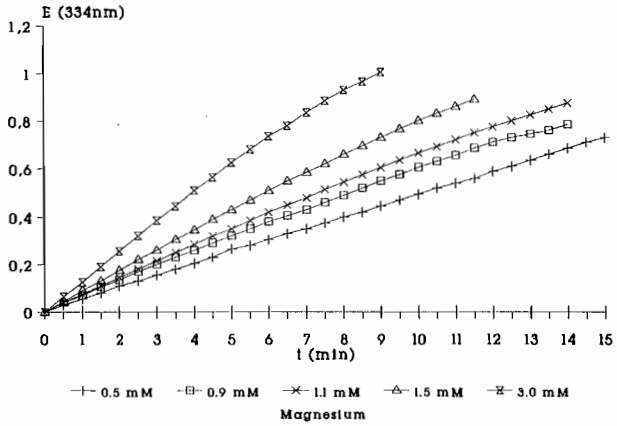


Fig. 1: Time course of hexokinase/glucose-6-phosphate dehydrogenase reaction at different magnesium concentrations.

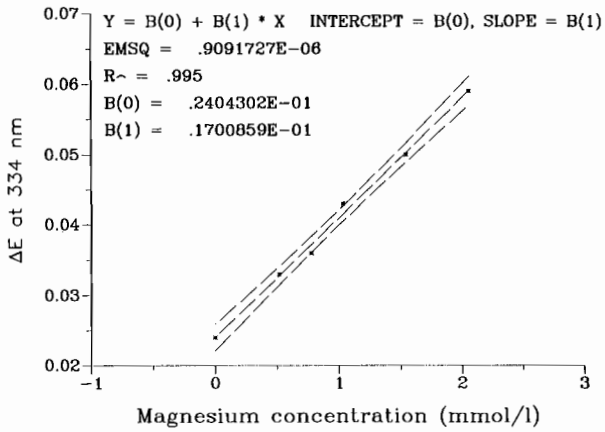


Fig. 2: Standard curve for kinetic determination of magnesium concentration in biological samples with the hexokinase/glucose-6-phosphate dehydrogenase reaction.

ent from DuPont) to 0.20 mmol/l (analyzed HPLC reagent from Baker) as determined by AAS. Further purification of the water from magnesium contaminactions was not successful.

### Effect of biochemical kations

Iron (FE), copper (Cu) and calcium (Ca) exert no effect in normal serum concentrations and in elevated serum concentration on determination of magnesium by hexokinase activation (fig. 3). Addition of 0.1 mmol/l. $CuSO_4$ is necessary for determination of magnesium by enzyme activation in EDTA-blood (fig. 4).

### Control sera

Tab. 2 shows the magnesium concentrations obtained in various control sera.

### Comparison of Methods

Table 3 lists the results of the Passing/Bablok regression analysis with 20 EDTA-plasma and 100 EDTA-whole blood samples using the kinetic method and the AAS for determination of magnesium. The mean is the arithmetic mean ± standard deviation in mmol/l. Figure 5 shows the graphic correlation of measured values.

Tab. 2: Assigned and measured magnesium concentrations in control sera.

| | assigned value | measured value by kinetic method |
|---|---|---|
| Control serum | | |
| Seronorm | 0.88 | 0.86 ± 0.14 |
| Kontrollogen L | 0.93 | 1.08 ± 0.08 |
| Beckman | | |
| Decision | 1.65 | 1.68 ± 0.10 |
| Titrisol | 2.06 | 2.12 ± 0.12 |

Tab. 3: Comparison of magnesium determination with kinetic method and AAS according to Passing/Bablok.

| Specimen | AAS mean | kinetic method mean | slope | intercept |
|---|---|---|---|---|
| Plasma | 0.87 ± 0.067 | 0.92 ± 0.07 | 1.00 ± 0.25 | 0.05 ± 0.23 |
| Blood | 1.41 ± 0.18 | 1.40 ± 0.18 | 1.04 ± 0.09 | –0.06± 0.14 |

## Discussion

Magnesium has recently attained clinical interest, since it is involved in many vital biochemical pathways. Magnesium is the physiologic calcium antagonist, and in such a role, it is used in treatment of acute myocardial infarction [6], arrythmias [7], and other heart diseases. In addition magnesium treatment is carried out in pregnancy [8], diabetes [9], sports medicine [10], and other diseases [11, 12, 13, 14], which are characterized by a magnesium deficiency.

For diagnosis of magnesium deficiency, this ion must be measured by the clinical chemist in all body fluids, including the erythrocytes, urine, and serum. In general, AAS is considered to be the reference method for determination of magnesium, however the price for the corresponding equipment is expensive. Chemical tests using chelating chromogens usually are considered to be labile to other che-

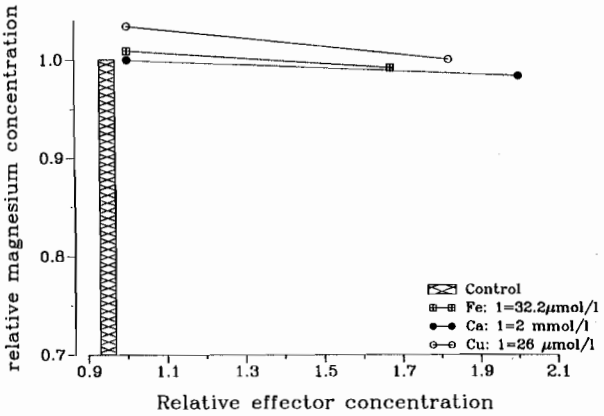


Fig. 3 Effect of iron, copper and calcium at notmal and elevated concentration on the kinetic determination of magnesium concentration in biological samples with the hexokinase/glucose-6-phosphate dehydrogenase reaction.

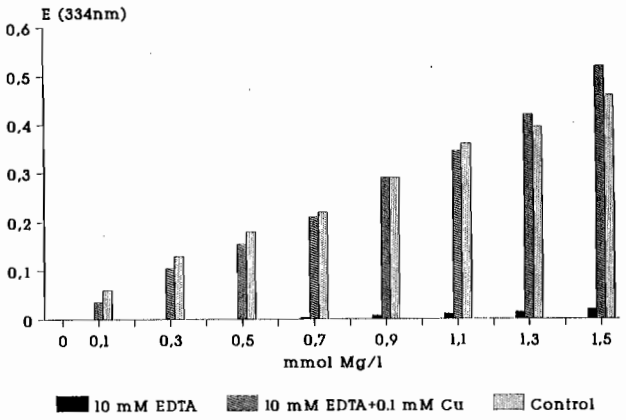


Fig. 4: Effect of 0.1 mmol/l $CuSO_4$ on kinetic determination of magnesium concentration in biological samples containing EDTA with the hexokinase/glucose-6-phosphate dehydrogenase reaction.

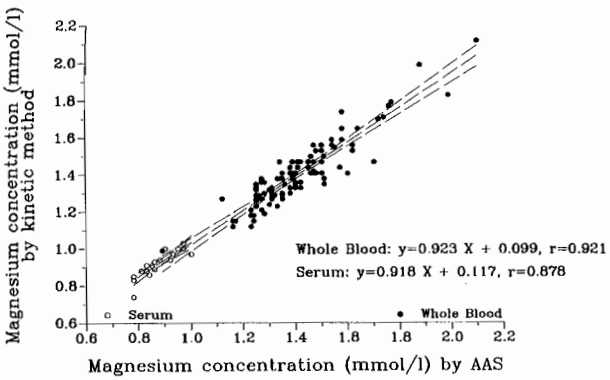


Fig. 5: Comparison of magnesium concentrations in blood samples obtained with atomic absorption spectroscopy (AAS) and with the kinetic method using the hexokinase/glucose-6-phosphate dehydrogenase reaction according to Passing/Bablok.

lating reagents, as well as to other divalent cations, which might interfere with magnesium. Tests for magnesium determination based on enzyme activation are known for some 30 years [15], and in recent reports some test modifications have been described, which seem to establish these enzymatic tests as routine methods [16, 17]. There exist numerous enzymes, which are directly or indirectly activated by magnesium. The most widely used enzyme combination in clinical chemistry, which uses magnesium is HK and G-6-P-DH. This enzyme combination is used for example in glucose and creatine kinase determination. An alternative for HK seems to be glucokinase (GK), which was originally used by *Tabata* et al. [16] and *Fossati* and al. [17]. In our experiments HK seemed superior to GK, since the corresponding specific price per enzyme unit is lower than in case of GK. In addition, the HK based test assay is linear up to 3 mmol/l magnesium (fig. 2). Use of GK in the test assay would only yield similar linearity with a higher enzyme concentration, and thus increase the price of test.

Chelating compounds are usually detrimental to the determination of magnesium based on an enzyme reactivation. In the original method from *Tabata* et al. [16]. EDTA was used as stopping reagent, since it effectively removes magnesium from the ATP-Mg-complex. On the other hand, EDTA-blood would be a primary choice for specimen collection when magnesium concentration is of clinical interest. The simultaneous measurement of magnesium concentration in whole blood and in the corresponding plasma allows the calculation of magnesium concentration in the erythrocytes, when the hematocrit is known. This approach has several advantages if compared to a purification and corresponding hemolysis of packed erythrocytes [18]. EDTA blood is usually collected from all patients for determination of blood parameters, such as hematocrit. The rest of the specimen could be used for magnesium measurement. For neutralization of EDTA copper might be used. Copper binds to EDTA with a binding constant of $10^{18.8}$, in contrast to magnesium, which is characterized by a binding constant of $10^{8.7}$ [19]. Addition of Cu effectively prevents inactivation of magnesium dependent activation of hexokinase as seen in fig. 4.

Due to the kinetic method of magnesium determination lipemic samples as well as hemolytic samples and samples with different protein concentration might be used without interference. It is clear, however, that a slight hemolysis does not interfere with the true magnesium concentration in plasma, since the additional magnesium transferred from hemolytic red blood cells to the plasma is negligible (100 mg Hb in plasma increase plasma magnesium concentration by about 0.01 mmol/l). Other biochemical effectors, such as Fe, Ca, and Cu do not interfere with determination of magnesium (fig. 3).

The presented method is precise and delivers accurate results. As judged by measurement of magnesium concentration in control sera, its coefficient of variation is located between 5 % and 8 % in the day-to-day test. It is, of course, very difficult to pipette 5 µl of plasma by hand. An automation of the method would without doubt decrease imprecision.

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