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Precision of the Magnesium Determination in Mononuclear Blood Cells and Erythrocytes

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Objective: Establishing the analytical variation and reproducibility of the intracellular magnesium (Mg) assay in mononuclear blood cells (MBC) and erythrocytes (RBC).
Design and Methods: We assessed the analytical variation of the several determination steps, and the reproducibility for the complete intracellular Mg-assay (combination of preanalytical, analytical, and biological variation). The influence of platelets was determined by comparing Mg concentrations obtained from heparinized blood and defibrinated blood.
Results: Coefficients of variation of the several determination steps used in the MBC- and RBC-assay were ≤5.4%. The overall analytical variation was 5.0–6.8%, and reproducibility of the complete Mg-assay 11.6–14.0%. Mg measurements in MBC (expressed as fmol/cell) obtained from heparinized blood showed significantly higher values than those obtained from defibrinated blood.
Conclusion: This is the first study to describe in detail reproducibility data for the individual steps in the overall procedure to measure intracellular magnesium. It is shown that results obtained in daily practice should be interpreted with care. Moreover, the removal of platelets is essential in the determination of Mg in MBC.

KEY WORDS: intracellular; reproducibility; leucocytes; monocytes; magnesium deficiency.

Introduction

Magnesium in the human body is mainly located intracellularly (Mg_{intra}), and it is after potassium the second most abundant intracellular cation. Serum magnesium contributes for <1% to the total amount in the body and its function as a marker for magnesium deficiency is doubtful (1). Therefore, an increasing interest can be noticed in the measurement of its intracellular concentration. Muscle or bone biopsies seem to be good samples but are not suited for routine measurements. Mononuclear blood cells (MBC) and erythrocytes (RBC) are more easy to obtain, but opinions about the clinical impact of these Mg parameters are not uniform. However, for a good interpretation of the relevance of determining Mg_{intra}, the precision of all elements of the assay, including the preanalytical ones, first must be established. For example, a MBC suspension obtained from heparinized blood is often contaminated with platelets (2,3). Although several studies about the diagnostic value, and relation of Mg_{intra} to other Mg parameters already have been published, until now no thorough study about the precision of the whole assay of Mg_{intra} has been described. A few authors have presented results from reproducibility measurements, but those were confusing and not always complete (2,4–7). The studies of Urdal *et al.* (8) and Schwinger *et al.* (9) provided more interesting data, but did not cover all aspects either.

Therefore, we established the analytical variation of the magnesium determination in MBC (expressed as fmol/cell and as μmol/g protein) and RBC (fmol/cell and as μmol/g dry weight), by measuring the within-day and day-to-day reproducibility of the cell count, dry weight, Mg, and protein measurements. Moreover, the within-day and day-to-day reproducibility were assessed of the complete intracellular Mg-assay (combination of preanalytical, analytical, and biological variation) in MBC and RBC obtained from heparinized blood. Since this type of sample may lead to interference from thrombocyte Mg at the measurement in MBC, we compared Mg_{intra} results obtained from heparinized blood with those from defibrinated blood as well.

Material and methods

Technical part

Blood samples, either 10 mL heparinized blood and/or 20 mL defibrinated blood, were obtained from healthy laboratory employees between 9 and 10 AM. Volunteers had their regular breakfast but did not

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use Mg supplements. Evacuated 10 mL lithium heparin tubes (15 U/mL) were obtained from Terumo (Leuven, Belgium). Tubes to prepare defibrinated blood (evacuated 10 mL tubes containing 0.8 g polystyrene granules) were obtained from Becton Dickinson (Etten Leur, The Netherlands). After defibrination both heparinized blood and defibrinated blood were treated identically.

To isolate MBC and RBC from whole blood, the blood samples were diluted with an equal amount of a phosphate buffered-saline solution (PBS; Na 160 mmol/L, H_2HPO_4 1.3 mmol/L, HPO_4 9.2 mmol/L, Cl 140 mmol/L), and layered over four tubes each containing 4 mL density gradient separation liquid (Lymphoprep, Nycomed, Norway). The tubes were centrifuged (400 × g, 35 min) and both the MBC and RBC fractions were pooled.

MBC were washed twice with PBS, and the final pellet was resuspended in 4.5 mL PBS. Of this, 0.5 mL was used for cell count and leucocyte differentiation. The remaining 4.0 mL MBC suspension was centrifuged (600 × g, 10 min), the pellet lysed with 1.0 mL distilled water, and stored at −20 °C until magnesium and protein were determined.

Of the isolated RBC 1.0 mL was washed three times with CsCl, 155 mmol/L, pH = 7.4 (600 × g, 10 min). For cell counting 100 μL was diluted with 400 μL PBS; 200 μL was lysed with 800 μL distilled water. The lysate was stored at −20°C until Mg and dry weight were determined.

Mg measurements of the cell lysates were performed by Atomic Absorption Spectrophotometry (PE2100, Perkin Elmer, Überlingen, Germany). The protein concentration of the MBC lysate was measured photometrically using Coomassie Brilliant Blue (Microprot, Oxford Labware, USA). Cell count was performed by a Bayer-H3-system (Bayer, Tarrytown, NY, USA), and the dry weight of the RBC lysate was measured by evaporating water (95°C, 60 min) from 100 μL lysate in preheated and weighed 1.0 mL glass tubes.

Experimental setup

To assess both the analytical variation and the reproducibility of the complete intracellular Mg-assay (a combination of the preanalytical, analytical, and biological variation) in MBC and RBC, the following experiments were performed.

The within-day *analytical variation* (expressed as $CV_{within\text{-}day}$) was determined by drawing 10 tubes of heparinized blood from one healthy volunteer. Isolated cells were pooled and all measurements (Mg, protein, cell count, and dry weight) were performed 10 times.

The day-to-day *analytical variation* (expressed as $CV_{day\text{-}to\text{-}day}$) was calculated by subtracting the within-day analytical variation from the overall analytical variation (CV_{all}) (see Equation 2). The latter was determined by drawing ten tubes of heparinized blood from two healthy volunteers each. Isolated cells were pooled per volunteer, and divided into ten aliquots, which were measured every next 10 days, with a 2-day break between day 5 and day 6. Aliquots used for cell count of RBC were stored at +4 °C, and aliquots used for Mg, protein and dry weight determinations were stored at −20 °C. Because MBC cannot be stored, the overall analytical variation of the cell count of MBC was approximated by using a commercial control sample (Parameter Control Low, Baker BV, Deventer, The Netherlands).

The within-day *reproducibility of the complete Mg-assay* (expressed as $CV_{within\text{-}day}$) was determined by drawing ten tubes of heparinized blood from one healthy volunteers. All ten blood samples were worked up separately.

The day-to-day *reproducibility of the complete Mg-assay* (expressed as $CV_{day\text{-}to\text{-}day}$) was calculated by subtracting the within-day reproducibility from the overall reproducibility (CV_{all}) (see Equation 2). The latter was determined by drawing 1 tube of heparinized blood from two healthy volunteers each, during 10 days, with a 2-day break between day 5 and day 6. Cells were isolated immediately and all parameters were measured on the day of sampling.

Comparison between the Mg concentration in MBC obtained from heparinized blood and defibrinated blood was performed by drawing 10 mL heparinized blood and 20 mL defibrinated blood from 17 healthy volunteers. Cells were isolated, lysed, and stored until all samples were collected.

Calculations

Mg in MBC was expressed as fmol/cell and μmol/g protein. Mg in RBC was expressed as fmol/cell and μmol/g dry weight.

Coefficient of variation (CV) of ten measurements was calculated as the standard deviation divided by the mean value. The CV of a ratioed unit (*e.g.*, fmol/cell) was calculated as follows (10):

$$CV_{fmol/cell} = \sqrt{CV_{MgD}^2 + CV_{CC}^2 + CV_{MgD}^2 \cdot CV_{CC}^2} \quad [1]$$

with, MgD the Mg determination in the cell lysate and CC the cell count. The day-to-day analytical variation or reproducibility of the complete Mg-assay was calculated as follows:

$$CV_{day\text{-}to\text{-}day} = \sqrt{CV_{all}^2 - CV_{within\text{-}day}^2} \quad [2]$$

In case of experiments performed in duplicate, the calculated CVs were averaged.

Statistical analysis of the difference between the Mg_{intra} concentration in the two different sample types was performed by a paired *t*-test.

All procedures followed were in accordance with the rules laid down in the Helsinki Declaration of 1975, as revised in 1983.

TABLE 1
Review of the Analytical Variation of the Several Determination Steps of the Intracellular Mg-Assay

	MBC						RBC					
	$CV_{within\text{-}day}$ (%)			$CV_{day\text{-}to\text{-}day}$ (%)			$CV_{within\text{-}day}$ (%)			$CV_{day\text{-}to\text{-}day}$ (%)		
Author	Mg	CC	Prot	Mg	CC	Prot	Mg	CC	DW	Mg	CC	DW
Elin	3.0	8.7										
Martin	<2.0	5–10	3.4									
Schwinger	1.7[a]	1.7[a]		2.4[b]	2.0[b]			0.9[a]			1.7[b]	
Urdal				5.3[c]		11.0[c]						
Reinhart				3.6[d]								
This study	1.6[e]	2.6	1.7	4.4	1.3	2.6	1.5	2.1	1.3	3.3	3.3	5.4

Mg = magnesium measurement; CC = cell count; Prot = protein measurement; DW = dry weight determination.
[a]Intraassay (n = 10).
[b]Interassay (n = 10).
[c]n = 12.
[d]n = 10.

Results

ANALYTICAL VARIATION

Within-day reproducibility measurements of the several determination steps of the Mg-assay resulted in CVs <2.7%. CVs of the day-to-day reproducibility were all <5.0%, with the exception of the dry weight determination (duplicate) of RBC, for which calculated mean CV was 5.4% (Table 1). From all measurements one result, the Mg concentration of the RBC lysate on day 7, was rejected. This value deviated more than 20% from the mean value based on the other nine concentrations.

Calculated CVs of the ratioed units based on the CVs of the numerator (Mg concentration in cell lysates) and denominator (cell count, protein concentration, or dry weight) are presented in Table 2. The overall CV of the analytical variation of the 4 Mg parameters ranges from about 5.5% to 6.8%.

REPRODUCIBILITY OF THE COMPLETE INTRACELLULAR MG-ASSAY

Results are presented in Table 2. The $CV_{within\text{-}day}$ of the Mg-assay in RBC was about 4.5%, and in MBC 8.0%. The $CV_{day\text{-}to\text{-}day}$ ranged from 9.0–11.5%, and the CV_{all} was for both types of cells more or less comparable, 12%, with the exception of MBC (μmol/g prot) which CV_{all} was found to be 14.0%.

HEPARINIZED BLOOD VERSUS DEFIBRINATED BLOOD

In Figures 1 and 2 the differences between the Mg concentration of MBC obtained from heparinized blood, and the Mg concentration of MBC obtained from defibrinated blood are plotted against the mean intracellular Mg concentration of these two different sample types (11). The results of a paired t-test are presented in the legend of each figure. When expressed as fmol/cell, 16 of the 17 heparinized blood samples resulted in a higher intracellular Mg concentration when compared with the simultaneously drawn defibrinated blood samples. This observation was found significant ($p < 0.001$). When expressed as μmol/g prot the mean difference between the two sample types was positive (4.6 μmol/g prot) too, but not significant.

Discussion

Since in clinical chemistry and medicine an increasing interest in the measurement of the Mg concentration of MBC and RBC can be observed,

TABLE 2
Analytical Variation and Reproducibility Results of the Complete Mg-Assay in Mononuclear Blood Cells and Erythrocytes

	Analytical Variation			Reproducibility of the Complete Mg-Assay		
	$CV_{within\text{-}day}$ (%)	$CV_{day\text{-}to\text{-}day}$ (%)	CV_{all} (%)	$CV_{within\text{-}day}$ (%)	$CV_{day\text{-}to\text{-}day}$ (%)	CV_{all} (%)
MBC (fmol/cell)	3.0	4.7	5.5	8.1	9.0	12.1
MBC (μmol/g prot)	2.3	5.2	5.7	8.0	11.5	14.0
RBC (fmol/cell)	2.6	4.3	5.0	4.0	10.9	11.6
RBC (μmol/g dry weight)	2.0	6.5	6.8	4.7	11.3	12.3

Presented CVs of the analytical variation are calculated according to Equations 1 and 2, based on the CV of ten Mg determinations, and the CV of ten determinations of the protein concentration, dry weight or cell count. $CV_{within\text{-}day}$ and CV_{all} (both mean of two) representing the reproducibility of the complete Mg-assay are both based on ten complete Mg determinations, and $CV_{day\text{-}to\text{-}day}$ was calculated according to Equation 2.

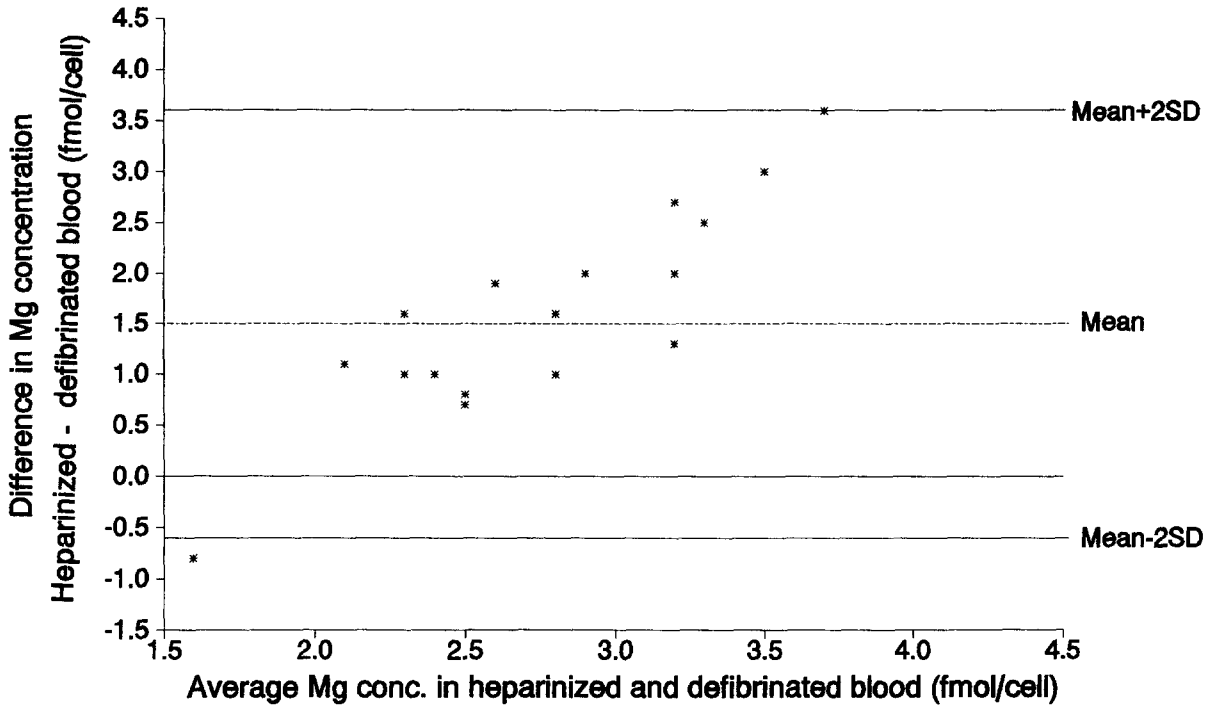

Figure 1 — Difference between Mg measurements in MBC obtained from heparinized, and defibrinated blood. Mg concentration expressed as fmol/cell. Paired t-test: $t = 6.36$, $p < 0.001$.

knowledge of the precision of the technique is essential. Therefore, this study about the reproducibility of the used analytical methods as well as the complete Mg-assay was performed. Although reproducibility measurements about intracellular Mg-assays are scarce, some comparison with other authors is possible.

Analytical variation

In Table 1 the reproducibility measurements of the several determination steps of the Mg-assay in MBC are compared with those from other authors. Elin *et al.* (4) reported a $CV_{within\text{-}day}$ for the Mg measurement and cell count, which was much higher than our results, but the reported $CV_{within\text{-}day}$ values of Martin *et al.* (2) and Schwinger *et al.* (9) corresponded better.

Urdal *et al.* (8) measured the Mg and protein concentration in MBC lysate on 12 different days. This resulted in an analytical $CV_{day\text{-}to\text{-}day}$ of 5.3% and 11%, respectively. As an average we found a comparable precision of the Mg determination in MBC lysate, but our mean $CV_{day\text{-}to\text{-}day}$ of the protein assay was much lower, 2.6%. The $CV_{day\text{-}to\text{-}day}$ of the Mg determination in stored MBC lysates reported by Schwinger *et al.* (9) was only 2.4%. However, this low value was presented as the interassay CV without further explanation.

The contribution of the within-day analytical variation of the ratioed unit to the total analytical variation was about half that of the day-to-day analytical variation (Table 2). Obtained values were comparable with the precision established by Deuster *et al.* (6) (CV 2.2%), who determined the within-run precision of the Mg-assay in RBC by six replicate analyses of one sample from six individuals.

Isolated MBC deteriorates during storage, which hampers cell counting of one sample on consecutive days. To overcome this problem we used a commercial control sample for establishing the CV_{all} of the cell count. After calculating the ratioed CV of the intracellular Mg concentration (fmol/cell) with Equation 1, and subtracting $CV_{within\text{-}day}$ (Equation 2) we found a $CV_{day\text{-}to\text{-}day}$ of 1.3%. We realize that this solution of using a commercial control sample is not ideal and presumably leads to an underestimated precision of the cell count, 1.3% ($CV_{day\text{-}to\text{-}day}$) versus 2.6% ($CV_{within\text{-}day}$). However, Schwinger *et al.* (9) were able to present both intra-assay and inter-assay CVs of the cell count of lymphocytes 1.7% and 2.0%, respectively (see Table 1). Unfortunately, no information about storage conditions was given.

For the day-to-day analytical variation of the Mg-assay in MBC, expressed as μmol/g prot, we found a value much lower than Urdal *et al.* (8), 5.2% versus 16%. Based on their reported values of the precision of the Mg and protein assay (Table 1), we were not able to calculate a ratioed CV of 16%. Therefore, we think this high value was obtained by calculating the final intracellular Mg concentration (μmol/g prot) daily, and using these multiple results for establishing the $CV_{day\text{-}to\text{-}day}$.

Reproducibility of the complete Mg-assay

As expected the $CV_{within\text{-}day}$ is smaller than the $CV_{day\text{-}to\text{-}day}$ (Table 2). However, the $CV_{day\text{-}to\text{-}day}$ of

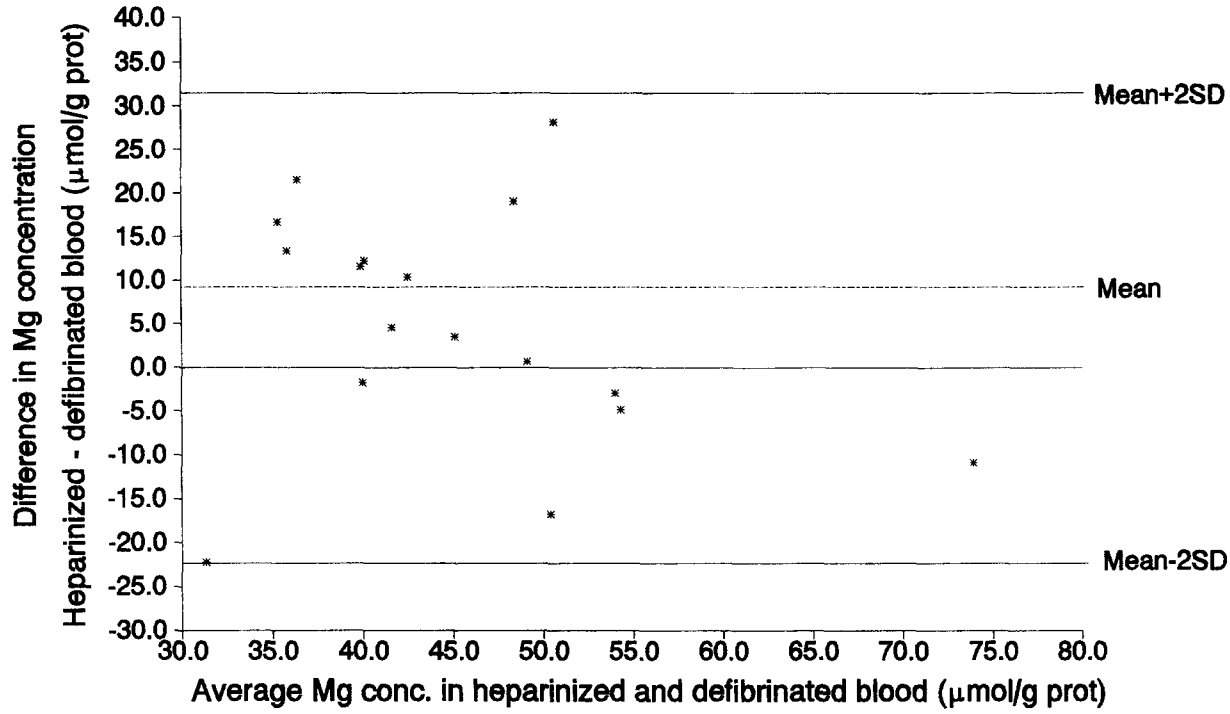

Figure 2 — Difference between Mg measurements in MBC obtained from heparinized and defibrinated blood. Mg concentration expressed as μmol/g protein. Paired t-test: $t = 1.45$, $p < 0.16$.

the Mg-assay in RBC is twice the $CV_{within\text{-}day}$, while the difference between $CV_{day\text{-}to\text{-}day}$ and $CV_{within\text{-}day}$ in MBC is only 1–3%. A possible explanation could be a very large contribution of the MBC isolation procedure to the inaccuracy, which is comparable for both the $CV_{within\text{-}day}$ and $CV_{day\text{-}to\text{-}day}$. As a result the $CV_{within\text{-}day}$ will be relative high (8%) and the difference between $CV_{within\text{-}day}$ and $CV_{day\text{-}to\text{-}day}$ of the Mg-assay in MBC reduced. In Table 3 our reproducibility measurements of the complete assay are compared with those of others. Reinhart *et al.* (5) determined the within-day reproducibility of the

TABLE 3
Review of Reproducibility Results of the Complete Mg-Assay in Mononuclear Blood Cells and Erythrocytes

Author		MBC		RBC	
		$CV_{within\text{-}day}$ (%)	$CV_{day\text{-}to\text{-}day}$ (%)	$CV_{within\text{-}day}$ (%)	$CV_{day\text{-}to\text{-}day}$ (%)
Reinhart	fg/cell	3.0[a]			
Martin	fmol/cell	8.8[b]	22.0[c]		
	μmol/g prot	12.0			
Gallager	nmol/10^6 cells	3.7[d]		5.8[d]	
Schwinger	fmol/cell	5.7[e]		3.5[e]	
Elin	fmol/cell		6.0, 17.9[f]		<5.0[f]
Urdall	fmol/cell		12.0[g]		
	μmol/g prot		12.0[h]		
Deuster	μg/g Hb			3.3[i]	
This study	fmol/cell	8.1[j]	9.0	4.0	10.9
	μmol/g prot	8.0[j]	11.5		
	μmol/g dry weight			4.7[j]	11.3

[a] $n = 10$.
[b] Duplicate analysis of samples from 24 subjects.
[c] Two samples with an interval of 7 days from ten subjects.
[d] Duplicate analysis of 15 specimens.
[e] Ten identical samples taken from five persons, from which lymfocytes were isolated.
[f] $n = 5$.
[g] $n = 9$, and 32% when $n = 12$.
[h] $n = 12$.
[i] $n = 6$.
[j] $n = 10$.

Mg-assay in MBC (expressed as fg/cell), too. They made ten cell isolates on the same day and assayed those as ten separate specimens. In our opinion, their CV (3.0%) is very low. In our study, the within-day analytical variation was already 3.0%. Martin *et al.* (2) determined the $CV_{within\text{-}day}$ of the Mg-assay in MBC by drawing a second sample from the same subject later on the day. Gallager *et al.* (7) assessed the precision of the entire assay by duplicate analyses of 15 specimens.

The day-to-day reproducibility of the complete Mg-assay was more or less comparable for both types of cells (Table 2). Elin *et al.* (4) reported lower values (<5.0% and 6.0%), but also a very high CV (17.9%), based on measurements performed during 5 days (Table 3). Urdal *et al.* (8) reported CVs for Mg in MBC comparable with ours. The $CV_{day\text{-}to\text{-}day}$ reported by Martin *et al.* (2) was high. They measured Mg in MBC (fmol/cell) in ten subjects twice, with 6 days in between.

The overall reproducibility of the complete assay varies from 11.6% to 14.0% (Table 2). Based on these values, and the CV_{all} of the analytical variation, it can be concluded that the contribution of the preanalytical (blood drawing and cell isolation) and biological variation was about 50% (45–59%) of the overall reproducibility of the complete Mg-assay in both MBC and RBC. Reported values for the intraindividual coefficient of variation are 18.1% and 7.8% (12), and 18.5% and 3.4% (7) for MBC and RBC, respectively. However, the measurements of Elin *et al.* (12) were performed five times with an interval of 5 months, Gallacher *et al.* (7) collected blood at regular intervals during 20 weeks, and our experiment took only 2 weeks. Biological change assessed by Martin *et al.* (2) and Schwinger *et al.* (9) was based on a period of 1 week. The former authors reported an intrasubject CV for Mg measurements in MBC (fmol/cell) of 22%, and the latter used three consecutive samples obtained during that week from 12 persons, which resulted in CVs of 4.8% and 5.9% for lymphocytes and RBC, respectively.

Heparinized blood versus defibrinated blood

From the results presented in Figures 1 and 2, it can be concluded that Mg measurements in MBC obtained from heparinized blood result in higher values than in MBC obtained from defibrinated blood, due to the presence of platelets. When expressed as fmol/cell the Mg concentrations measured in both sample types are significantly different ($p < 0.001$). Martin *et al.* (2) mentioned the contamination of platelets as a possible contribution to the analytical error of the Mg-assay too. In a subsequent letter, Kemp *et al.* (3) stated that ignoring the contribution of these contaminating platelets can lead to an overestimation of the Mg concentration of up to 75%. Elin *et al.* (4) discerned this problem and removed the platelets by repeatedly washing the blood sample before the isolation procedure was started, and Schwinger *et al.* (9) introduced an extra centrifugation step before layering the buffy coat on the gradient. We think our method of using defibrinated blood is less time consuming and easier to perform. Anyhow, removal of the platelets is an essential step in the Mg-assay in MBC, in which washing of the already isolated MBC does not result in the intended result.

In conclusion, the CVs of the reproducibility of the complete Mg-assay are rather high. Since the presented CVs are obtained using blood of healthy volunteers, these results cannot be extrapolated for patients with abnormal Mg_{intra} concentrations. Improvement of the method, or development of new methods (*e.g.*, determination of intracellular ionized Mg) seems to be necessary. Removal of platelets should be an essential step in the isolation procedure of MBC, and Mg_{intra} concentrations obtained in daily practice should be interpreted with care in view of the relative high coefficient of variation.

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