CLIN. CHEM. 28/10, 2149-2152 (1982)

Total Calcium and Magnesium Determined in Serum with an Automated Stopped-Flow Analyzer

M. A. Koupparis,[1] E. P. Diamandis,[1] and H. V. Malmstadt

We describe the measurement of total calcium and magnesium in serum with an automated microcomputer-controlled stopped-flow analyzer. The calcium method is based on the cresolphthalein complexone procedure, with 2-amino-2-methyl-1-propanol as the alkalinizing agent. The assay, performed on 60-fold prediluted samples, requires 50 μL of serum. Absorbance is measured at 580 nm for 1 s, after a 5-s delay. Response is linearly related to concentration up to 5 mmol/L; analytical recovery averaged 97.8%. Within-day CVs were 0.7 to 1.5%, day-to-day CVs 1.8 to 2.5%. Results compared well with those by continuous-flow Technicon SMA II method. A sample throughput of as many as 260 samples per hour is possible. The magnesium determination, a complexometric procedure, involves magnesium/calmagite complex in an alkaline reagent mixture and ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid to eliminate calcium interference. Prediluted serum samples are used (100 μL of serum diluted 25-fold), and absorbance at 520 nm is linear with concentration to 50 mg/L. Within-run CVs were 0.5 to 1.1%, and day-to-day 1.3 to 3.8%; analytical recovery was 99.3%. Results compared well with those by atomic absorption spectrometry ($r = 0.994$). A delay time of 10 and a measurement time of 2.5 s allows for a throughput of as many as 180 samples per hour.

Determination of total calcium in serum is so routine in the clinical laboratory that rapid and accurate automated methods are important. Various methods have been developed: precipitation techniques, direct EDTA titrations, atomic absorption spectrometry, ion-selective electrodes, and spectrophotometry. Most manufacturers' manual diagnostic kits and methods for automated instrumentation rely on the complexometric reaction between calcium and *o*-cresolphthalein complexone. The first automatic calcium determination, introduced by Kessler and Wolfman (*1*), required dialysis of the serum, to obviate protein interference, and a solution of pH 12 to minimize interference from magnesium; the buffering agent was diethylamine. The method was much improved (*2*) by using 8-quinolinol to complex magnesium in a more suitable buffer of higher capacity to stabilize the pH and automating this method. Recent variations in this procedure provide more stable absorbance values and reproducible results by modifying the buffering agent used (*3–5*). Moorehead and Biggs (*5*) substituted 2-amino-2-methyl-1-propanol for diethylamine, which results in a solution of pH 10 to give a lower blank and more stable final absorbance. The usefulness of dialysis in the determination of calcium is questionable; Zak et al. (*6*) showed that a simplified procedure without dialysis yields reliable results.

Department of Chemistry, University of Illinois, Urbana, IL 61801.

[1] Present address: Laboratory of Analytical Chemistry, University of Athens, 104 Solonos St., Athens (144), Greece.

Address correspondence to H. V. M.

Received Dec. 1, 1981; accepted June 18, 1982.

The method presented here is based on the mixing of prediluted serum samples with a reagent mixture similar to the improved reagent system by Moorehead and Biggs (*5*) and the use of our relatively simple microcomputer-controlled stopped-flow analyzer (*7*).

We also adapted to the stopped-flow analyzer a procedure for the determination of magnesium in serum, the dye-binding complexometric method proposed by Gindler and Heth in 1971 (*8*). This method is based on formation of a Mg–calmagite complex in an alkaline reagent mixture containing calmagite, *p*-nonylphenol, and polyvinylpyrrolidone. Calcium interference is eliminated by preferential combination with ethylene glycol (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (*9*) and heavy metals are complexed by cyanide ions. The method was introduced commercially by the Pierce Chemical Co., Rockford, IL 61105, and has been adapted also to a centrifugal analyzer (*10*).

Materials and Methods

Apparatus

The stopped-flow analyzer designed in our laboratory (*7*) is used for equilibrium or reaction-rate methods, with accurate and precise results. It is a combination of a reagent/sample-handling system and a simple photometric unit with an 18-μL flow cell. The two syringes of the reagent/sample unit are driven by a double-acting air cylinder and used for automatic aliquoting and mixing of prediluted serum samples and reagent and delivery of the mixed solution into the 1-cm cuvet. Two three-way valves with a low dead volume are used for automatic control of solution flow. A low dead-volume X-Y mixing chamber, made of Teflon, is used to rapidly and efficiently mix the two solutions. A check valve placed after the flow-cell output provides a back pressure during the delivery operation and avoids the generation of fine bubbles.

Tungsten and deuterium light sources (EU-701; Heath Co., Benton Harbor, MI 49022) are used and three-cavity, 1-in.-diameter interference filters (Ditric Optics, Inc., Marlboro, MA 01752) with about 10-nm bandpass are used for wavelength isolation. A photomultiplier module (Heath EU-701 series) with a high-voltage power supply is used as the detector. The system also has an automatic shutter for 0 and 100% transmittance settings. Each syringe delivers 150 μL of prediluted sample or reagent, and four flushes are used to prevent sample-to-sample carryover.

We used an AIM 65 (Rockwell International, Anaheim, CA 92803) microcomputer to interface the whole system. A 20-character visual display and a thermal printer incorporated in the microcomputer system provide user communication. Machine-language routines and BASIC programs are loaded from a cassette tape recorder. Investigative programs are used in optimizing rate and equilibrium methods, and interactive routine or dedicated programs are used for routine analysis.

Interference filters, 580 nm for calcium and 520 nm for magnesium determinations, are used in the photometric unit. All measurements were made in a laboratory maintained near 25 °C.

To dilute the samples we used an automatic pipette (Micromedic Systems, Inc., Horsham, PA 19044). The comparison studies for calcium were done with an SMA II multichannel continuous-flow analyzer (Technicon Instruments, Tarrytown, NY 10591) and the cresolphthalein complexone method. Magnesium results were compared with those obtained with an AA-475 atomic absorption spectrophotometer (Varian Associates, Palo Alto, CA 94303).

Reagents

All solutions were prepared in de-ionized water from reagent-grade materials.

Cresolphthalein complexone (CPC) stock reagent. Add 187.5 mg of *o*-cresolphthalein complexone (Sigma Chemical Co., St. Louis, MO 63178) to 150 mL of water in a 500-mL volumetric flask. Add 4.2 g of 8-quinolinol and 10 mL of concentrated HCl to 200 mL of water, and transfer this to the volumetric flask. Dilute to 500 mL with water and store in a polyethylene container.

Aminomethylpropanol stock reagent. Add 1.25 g of KCN to 150 mL of water in a 500-mL volumetric flask. Slowly add about 110 g of 2-amino-2-methyl-1-propanol (Aldrich Chemical Co., Milwaukee, WI 53202) and dilute the mixture to 500 mL. Store the solution in a polyethylene container.

Working reagent. Mix equal volumes of CPC and aminomethylpropanol stock reagents to form a red solution and store in an acid-washed glass bottle (CPC seems to permeate polyethylene container walls and permanently stain them a deep-yellow). Before preparing or storing any of the solutions, wash the glassware with dilute nitric acid to remove any traces of calcium. Rinsing all glassware with the working reagent prevents contamination.

Calcium standard stock solution, 25.0 mmol/L. Place 2.4970 g of $CaCO_3$ and 50 mL of water in a 2-L beaker. Add 5 mL of concentrated HCl and dissolve. Add about 750 mL of water and adjust the pH to 7.0 ±0.5 with 5 mmol/L NaOH. Transfer to a 1-L volumetric flask and dilute to the mark.

Calcium standard working solutions. 1.00, 2.00, 3.00, 4.00, and 5.00 mmol/L. Appropriately dilute the calcium stock solution with de-ionized water.

Calmagite stock reagent. Place 200 mg of calmagite (1-hydroxy-4-methyl-2-phenylazo-2-naphthol-4-sulfonic acid; Eastman Kodak Co., Rochester, NY 14650), 74.6 g of KCl, and 3.4 g of *p*-nonylphenol (Bion NE-9) and 31.4 g of polyvinylpyrrolidone (Bion PVP), both from Pierce Chemical Co., in a 1-L volumetric flask and dilute to volume with de-ionized water. Store in a polyethylene container.

Base reagent. Dissolve 1 g of KCN, 4.96 g of KOH, and 700 mg of EGTA (Sigma Chemical Co.) in 1 L of de-ionized water. Store in a polyethylene container.

Calmagite working reagent. Prepare freshly every day by mixing equal volumes of calmagite stock and base reagent. Store in a polyethylene container, which is kept closed when not in use.

Magnesium standard stock solution, 100 mg/L. Dissolve 1.835 g of $Mg(IO_3)_2 \cdot 4H_2O$ (Sigma) in 1 L of de-ionized water.

Magnesium standard working solutions, 10, 20, 30, 40, and 50 mg/L. Prepare with appropriate dilutions of the magnesium stock solution.

Saline diluent. 9 g/L sodium chloride solution.

Quality-control sera. Lyophilized control sera used were "Validate-Normal" and "Versatol-A" (General Diagnostics, Morris Plains, NJ 07950), "Moni-Trol I and II" (Dade Div., American Hospital Supply Co., Miami, FL 33152), and "Chem Trol" (Clinton Lab., Santa Monica, CA 90404). Patients' sera were used in evaluating the method.

Procedures

Determination of calcium. Standards or serum samples (50 μL) are prediluted with 3.0 mL of saline diluent in 5-mL plastic cups with an automatic pipette and then loaded on the turntable along with a blank (saline diluent). For each turntable (24 samples), include at least three standards and one control for recalibration. Into one channel is aliquoted the reagent solution, into the other channel an equal volume of diluted sample. The interactive routine equilibrium program is loaded from the cassette recorder into the computer's memory. The 0 and 100% transmittance are set during the execution of the program by using the blank and the reagent; the time parameters, along with the number of standards and samples, the number of flushes to change from sample to sample, and the number of runs to be averaged are requested from the operator. For calcium determination, the delay time is 5 s and the time for a measurement is 1 s (two 0.5-s integrations). The program then sequences through each standard, flushing the system between each standard, and prompting the operator for its concentration. After the calibration curve is calculated, the samples are measured and their calcium concentrations are calculated and printed.

A dedicated software program can also be used that contains all the required information, including the settings for time intervals and standard concentrations. Once the operator specifies the number of samples, all samples are analyzed automatically.

Determination of magnesium. The same procedure as for calcium is used, except that 100 μL of standards or samples is prediluted with 3.5 mL of saline. The delay time is 10 s and the measurement time 2.5 s (five 0.5-s integrations).

Correlation studies. We compared the results for calcium with those obtained with an automated air-segmented continuous-flow cresolphthalein procedure used in an SMA II analyzer. Results for magnesium were compared with those obtained with an atomic absorption spectrophotometer, for which 100 μL of standards or samples was prediluted with 5.0 mL of 2000 mg/L strontium solution and absorbance in an oxidizing air–acetylene flame was measured at 285.2 nm.

Results

Optimization of the Procedures

The reaction of calcium with CPC is complete in less than the dead time of the stopped-flow unit, so that an equilibrium procedure can be performed with as short as possible delay time. We used a delay time of 5 s to avoid the nonreproducible absorbance behavior noted when the working reagent is mixed with water. Use of 120-fold final dilution of the samples in the observation cell overcomes the need for dialysis to avoid any protein interference. The final concentrations (mmol/L) of the various components of the working reagent in the observation cell are: CPC 147, 8-quinolinol 1.5, HCl 60, aminomethylpropanol 620, and KCN 9.6.

The magnesium–calmagite reaction is also rapid, so a short delay time can be used. When Bion NE-9 and Bion PVP are included, the working dye reagent causes turbidity during the mixing with the samples. With a 5-s delay time, within-run precision was rather poor. Therefore, a delay time of 10 s and a measurement time of 2.5 s are used for routine analyses. The final dilution of samples for magnesium determination is 70-fold in the observation cell, and the final concentrations, per liter, of the reagent components are: calmagite 0.140 mmol, KCl 250 mmol, Bion NE-9 0.85 g, Bion PVP 7.85 g, KCN 3.8 mmol, KOH 22 mmol, and EGTA 0.46 mmol.

Analytical Variables

Linearity and stability of working curves. The working

Table 1. Day-to-Day Stability of Calcium Standards and Working Reagent

Calcium std., mmol/L	Absorbance		
	Day 1	Day 5	Day 10
1.00	0.145	0.158	0.161
2.00	0.296	0.305	0.307
3.00	0.442	0.455	0.454
4.00	0.589	0.604	0.607
5.00	0.753	0.764	0.767
Slope	0.1509	0.1511	0.1512
Intercept	−0.008	0.004	0.006
r	0.9998	0.99990	0.9998

Table 2. Effect of Delay Time on Magnesium Working Curve

Magnesium, mg/L	5-s delay time		10-s delay time	
	Absorbance [a]	CV, %	Absorbance [a]	CV, %
10.0	0.094	2.0	0.094	1.2
20.0	0.177	1.1	0.182	1.1
30.0	0.255	0.3	0.259	0.5
40.0	0.318	0.5	0.326	0.4
50.0	0.398	0.3	0.408	0.2
Slope	0.00749		0.00772	
Intercept	0.024		0.022	
r	0.9990		0.9991	

[a] Average of five measurements.

Table 3. Day-to-Day Stability of Magnesium Standards and Reagents

Magnesium std, mg/L	Absorbance [a]	
	Day 5	Day 10
10.0	0.094	0.094
20.0	0.178	0.182
30.0	0.255	0.248
40.0	0.315	0.314
50.0	0.396	0.391
Slope	0.00741	0.00726
Intercept	0.025	0.028
r	0.9990	0.997

[a] Average of five measurements. Values for Day 1 are given in Table 2, delay time, 10 s.

curve for determination of calcium is linear to 5 mmol/L. Typical results obtained for the working curve are shown in Table 1, which also shows results of studies of the stability of the reagents and standards. Except for the first standard, the precision (CV) for the absorbance values measured during this 10-day period was <2%. The CV for the slope of the working curve was <0.5%.

For the determination of magnesium, the working curve was linear up to 50 mg/L. Typical results for the working curve are shown in Table 2 for delay times of 5 and 10 s. The 5-s delay time shows good within-run precision for aqueous standard solutions but poor for serum samples, so we chose to use a 10-s delay time for routine analysis. Table 3 shows the day-to-day precision for the magnesium working curve in a 10-day period. The working reagent was prepared freshly every day. The absorbance values obtained and the slopes of the calibration curves show a CV of about 2%.

Precision. We evaluated within-run and day-to-day precision for calcium and magnesium determinations, using four commercial control sera (Table 4).

Analytical recovery. We checked the accuracy of the proposed methods by adding various known amounts of calcium or magnesium standard in control sera. For calcium, the analytical recovery ranged from 94 to 102%, with a mean of 97.8%. For magnesium, the recovery ranged from 94.5 to 107%, with a mean of 99.3%.

Sample throughput. When four flushes are used to change from one solution to another (flush cycle time, about 1.5 s), one measurement per sample, 1 s for the turntable position increment, and 0.5 s for the computer calculation and printing time, the sample throughput is 260 samples per hour for calcium and 180 for magnesium.

Comparison with Other Methods

The proposed method for determination of calcium was compared with the Technicon SMA II cresolphthalein continuous-flow method. Fifty serum samples obtained from a local hospital were analyzed by the two methods. Calcium values were in the range of 1.85 to 2.48 mmol/L. The mean difference obtained was ±0.08 mmol/L.

Magnesium results by the stopped-flow method were compared with those by atomic absorption spectrometry. Fifty samples in the range of 14.6 to 53.0 mg/L showed a correlation with a slope of 1.00, intercept 0.40, and $r = 0.994$.

These methods are based on fast reactions that exploit the advantage of the automated stopped-flow technique for obtaining rapid results. No deproteinization is required and only one reagent mixture is used.

The methods are sensitive and linear through the useful

Table 4. Precision of Determinations (n = 10 each)

	Ca samples [a]				Mg samples [b]			
	1	2	3	4	1	2	3	4
Within-run								
Mean, mmol/L	2.23	1.86	2.37	2.00	24.0	35.2	22.8	24.6
SD, mmol/L	0.016	0.028	0.019	0.020	0.17	0.18	0.25	0.12
CV, %	0.7	1.5	0.8	1.0	0.7	0.5	1.1	1.5
Day-to-day								
Mean, mmol/L	2.30	1.85	2.35	1.98	24.3	34.9	23.0	24.8
SD, mmol/L	0.048	0.046	0.042	0.040	0.9	0.7	0.3	0.4
CV, %	2.1	2.5	1.8	2.0	3.8	2.0	1.3	1.6

[a] Ca samples: 1, Validate; 2, Versatol-A; 3, Monitrol I; 4, Monitrol II. [b] Same as for calcium, except sample 4 is ChemTrol, and concentration is mg/L.

range, require only small volumes of specimens and reagents, and are precise and accurate for both aqueous standards and serum samples. The additional step of predilution of the samples is not a serious disadvantage since automatic diluters/dispensers are common instruments in routine laboratories.

The stopped-flow analyzer is an inexpensive, compact system, and it is easy to change from one analyte to another. Equilibrium and rate methods are easily performed with high sample throughputs and accurate and precise results. Measurements are obtained a few seconds after the samples are mixed with reagent.

We thank Mercy Hospital, Urbana, IL, for providing and analyzing the samples with SMA II. This work was supported in part by NIH grant no. PHS-5R01 GM 21984.

References

1. Kessler, G., and Wolfman, M., An automated procedure for the simultaneous determination of calcium and phosphorus. *Clin. Chem.* **10**, 686–703 (1964).

2. Gitelman, H. J., An improved automated procedure for the determination of calcium in biological specimens. *Anal. Biochem.* **18**, 521–531 (1967).

3. Fischl, J., and Schwartz, F., Modified "calcium base reagent" for simultaneous determination of serum calcium and inorganic phosphate with the "AutoAnalyzer." *Clin. Chem.* **18**, 1430–1431 (1972).

4. Baginski, E. S., Marie, S. S., Clark, W. L., and Zak, B., Direct microdetermination of serum calcium. *Clin. Chim. Acta* **46**, 49–54 (1973).

5. Moorehead, W. R., and Biggs, H. G., 2-Amino-2-methyl-1-propanol as the alkalizing agent in an improved continuous-flow cresolphthalein complexone procedure for calcium in serum. *Clin. Chem.* **20**, 1458–1460 (1974).

6. Zak, B., Salancy, J., Clark, W. L., et al., Accelerated, undialyzed procedure for serum calcium. *Advances in Automated Analysis, Technicon International Congress 1972*, Mediad Inc., White Plains, NY, 1973.

7. Koupparis, M. A., Walczak, K. M., and Malmstadt, H. V., Compact automated microprocessor-based stopped-flow analyzer. *J. Autom. Chem.* **2**, 66–75 (1980).

8. Gindler, E. M., and Heth, D. A., Colorimetric determination with bound "calmagite" of magnesium in human blood serum. *Clin. Chem.* **17**, 662 (1971). Abstract.

9. Schmid, R. W., and Reilley, C. N., New complexone for titration of calcium in the presence of magnesium. *Anal. Chem.* **29**, 264–268 (1957).

10. Khayam-Bashi, H., Liu, T. Z., and Walter, V., Measurement of serum magnesium with a centrifugal analyzer. *Clin. Chem.* **23**, 289–291 (1977).