

# Sensitive methods for the titrimetric micro-determination of biological calcium and magnesium

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**SYNOPSIS** New reagents containing high concentrations of urea are developed for micro-titration of calcium and magnesium, with ethylenediamine tetra-acetic acid (E.D.T.A.) as titrant and Corinth Ca (Plasmocorinth B) as indicator.

Magnesium is determined as the difference between calcium plus magnesium and calcium.

Quantitative aspects are studied, and accurate titration of untreated serum or urine is believed to be possible; precision is satisfactory.

The methods are simple, robust, and suitable for routine use.

Normal ranges are established for serum from blood donors of each sex. The mean serum calcium level for women is found to be lower than for men, while the mean magnesium contents are approximately the same. The latter appear to be somewhat lower than values found by flame emission spectrophotometry; in very good agreement with a mean value for plasma obtained by flame absorption spectrophotometry; and intermediate when compared with the values obtained by two other titrimetric procedures.

Most methods for the titrimetric determination of calcium and magnesium with E.D.T.A. stem from the original work of Schwarzenbach and Ackermann (1947), of Schwarzenbach, Biedermann, and Bangerter (1946), and of Biedermann and Schwarzenbach (1948). References up to 1956 to earlier work on the analysis of water and biological materials for these cations may be found in the studies of such authors as Horner (1955), Carr and Frank (1956), and Zak, Hindman, and Fisher (1956). Practically all methods of this nature have employed two indicators: eriochrome black T for calcium plus magnesium, and murexide for calcium determinations. Only in more recent years have other reasonably specific, stable, and sensitive dyes been described. For example, Yanagisawa (1955) and Kingsley and Robnett (1957) developed colorimetric methods for the determination of serum calcium using Corinth Ca (Plasmocorinth B), which is the disodium salt of 1-hydroxy-4-chloro-2, 2-diazobenzene-1, 8, dihydroxynaphthalene-3, 6-disulphonic acid, and is closely related in structure to the

eriochromes. Subsequently Kovács and Tárnoky (1960) reported a two-stage procedure using this dye; calcium is titrated first, and then magnesium after lowering the pH by adding hydrochloric acid and ammonia-ammonium chloride buffer. End-points are determined by visual observation.

This paper presents new reagents for methods which are believed to show improvements upon other similar methods previously described. The procedures are direct and simple to perform. Few reagents are required and these are cheap to make. The titrations are sensitive, free from annoying colour drift as detected by a galvanometer or colorimeter, and give sharp definitive end-points.

#### MATERIALS AND METHODOLOGY

A 24-hour specimen of urine was collected in a clean, polythene bottle containing 5 ml. of 10 M hydrochloric acid. Serum was used directly.

Specimens of normal blood were collected from donors at the end of regular donation. All collections were made during mid-morning and donors were instructed to have a light breakfast.

where possible. Use metal-free water and acid-washed glassware and store all reagents in polythene bottles.

**Calcium reagent** This is 0.2 M sodium hydroxide containing 25% urea. Dissolve 125 g. of urea in 100 ml. of M sodium hydroxide and some water, warm to room temperature and dilute to 500 ml. with water. Titrate the reagent and adjust it to have zero blank, as described later. Prepare freshly every four weeks, as the sensitivity of the titration tends to drop with increasing age of the reagent.

**Calcium plus magnesium reagent** This is approximately 0.25 M ammonium hydroxide containing 30% urea. Dissolve 150 g. urea in 350 ml. of water, add 7.1 ml. of fresh concentrated ammonia (S.G. 0.880), dilute to 500 ml. and mix. The pH of this mixture is about 10.9. Reduce the blank to zero as described later. This reagent is stable indefinitely at room temperature.

**Corinth Ca reagent** Dissolve 10 mg. of dye (available from Clinton Laboratories, 6010 Wilshire Boulevard, Los Angeles 36, California, U.S.A.) in about 50 ml. water, add one drop of concentrated hydrochloric acid and dilute to 100 ml. The acid dye is stable for at least six months when stored at about 5°C.

**E.D.T.A. 0.01 M** Dissolve 0.745 g. of E.D.T.A. disodium salt (British Drug Houses) in water and dilute to 200 ml. This is stable indefinitely at room temperature. The exact concentration is not critical and the weighing need not be accurate. Standardize the solution with fresh calcium and magnesium standards. The reagent is suitable for use with an Agla micrometer syringe (Wellcome Foundation, Ltd.) about 63  $\mu$ l. (315 units or divisions of the micrometer scale) being required for 0.25 ml. of a 10 mg. % calcium standard.

**Calcium standards** Oven-dry some calcium carbonate (British Drug Houses) overnight at 200°C. and after cooling in a desiccator over calcium chloride transfer to a tightly stoppered bottle and keep specifically for future standards. No further drying should be necessary. Wash 0.2500 g. into a 100 ml. volumetric flask with a small amount of water followed by sufficient approximately 5 M hydrochloric acid just to react with the carbonate. When the reaction is completed, dilute the solution to 100 ml. with water. This solution contains 100 mg. of calcium per cent. For a dilute standard, prepare an acid-washed flask by rinsing with 1% aqueous cetyltrimethyl-ammonium bromide (Cetavlon) followed by ion-free water. Dilute 10 ml. of strong standard in this flask and obtain a 10 mg. % calcium standard. Preserve with a few drops of chloroform. The standards usually keep for long periods. Check occasionally by titration with the E.D.T.A. reagent.

**Magnesium standards** Start with magnesium turnings (British Drug Houses, special for Grignard reactions). React 25 mg. with a minimum of approximately 5 M hydrochloric acid and dilute the solution to 100 ml. with water. For the working standard prepare a flask and dilute the strong standard 1 in 10 as before. The working standard contains 2.5 mg. of magnesium per cent. Preserve it with chloroform. Check occasionally against the standardized E.D.T.A.

**INSTRUMENTATION** The major part of the work presented was carried out using an E.E.L. titrator model A, which

employs magnetic stirring, in conjunction with an E.E.L. galvanometer of variable sensitivity; for the titration 4 ml. cylindrical vessels with an internal diameter of 1.4 cm. as supplied with the titrator may be used, but any flat-bottomed tube of about the same dimensions is just as suitable. The appropriate filter is one similar to the Ilford 607. Spectrophotometrically, the best wavelengths, giving maximum change in linear absorbance in going from bound to free dye, are found to be 590  $m\mu$  for the calcium plus magnesium titration and 600  $m\mu$  for calcium alone. The same filter is suitable for both titrations. Standardized E.D.T.A. solution is added from an Agla syringe; the usual fragile bent glass needle supplied with this is cut down, or alternatively a broken needle is used. To it is attached a short length of fine polythene tubing bent at right angles by gentle heating and drawn out at the tip to a fine bore by heating over a micro flame. No diffusion of E.D.T.A. solution is observed during titration.

**PROCEDURE FOR SERUM CALCIUM** Into a 4 ml. tube measure 0.25 ml. of serum. Add 2.5 ml. of calcium diluting reagent, followed by 0.5 ml. dye. Place on the titrator and start the magnetic stirrer. Run in about 85% of the anticipated quantity of E.D.T.A. (or add E.D.T.A. carefully until the first sign of a colour change) and stir for about one minute. Then add amounts of 5 units (1  $\mu$ l.), reading between each addition and continuing until no further significant change occurs in the galvanometer reading. Adjust the sensitivity continuously until approaching the end-point in order to keep the galvanometer spot towards the right-hand end of the scale. The titrations are sufficiently sharp to obviate the necessity for plotting the curves and locating end-points by extrapolation of the sloping and horizontal (or near-horizontal) portions of the curves—a time-consuming operation.

**PROCEDURE FOR SERUM CALCIUM PLUS MAGNESIUM** The quantities and technique are the same as for calcium, except that the appropriate diluting reagent is used.

**PROCEDURE FOR URINE** Measure the volume of the well-mixed 24-hour specimen and dilute one fortieth of it to 100 ml. with water (this is equivalent to diluting the whole 24-hour volume to 4 litres). Titrate 0.25 ml. (but not more) of the diluted urine with both reagents as for serum. In each case add the bulk of the E.D.T.A. as before. Equilibration at this stage is a little slower than with serum and adequate time (about two minutes) should be allowed before completing the titration.

**STANDARDIZATION** Use 0.25 ml. or other known volume of standard calcium or magnesium or calcium and/or magnesium, and titrate as for serum in the appropriate diluting reagent. External standards give the same results as internal ones for either serum or urine. Carry out standardizations at intervals. If any change is found in the volume of E.D.T.A. required, suspect the standard rather than the E.D.T.A., which appears to suffer little change. The standards are cross-checked with each other; for those referred to under 'Methodology' the number of units required for 0.25 ml. of magnesium standard should

equal 0.412 of the units for the calcium standard. If exactly 0.01 M E.D.T.A. were used, the volume required for a 10 mg. % calcium standard would be 313 units (62.2  $\mu$ l.) and for a 2.5 mg. % magnesium standard it would be 129 units (25.8  $\mu$ l.).

**BLANKING OF REAGENTS** Measure 2.5 ml. of diluting reagent into a titration vessel, add 0.5 ml. dye and titrate by adding very small quantities (3 to 5 units) of E.D.T.A. at a time. Calculate the number of microlitres of E.D.T.A. required to chelate the appropriate cation in the whole batch of reagent. Add the required volume, avoiding the neck of the bottle, mix thoroughly and re-titrate if considered necessary. Check the reagents for blank values at intervals and re-adjust if necessary. Urea usually contains traces of calcium and magnesium; the dye also may, but this is automatically allowed for in the adjustments.

#### RESULTS AND DISCUSSION

**RECOVERY EXPERIMENTS** In order to assess the quantitative aspects of the methods two experiments were designed. In the first of these, a pooled serum and three urines were titrated as described, both in the absence and presence of accurately added amounts of standard magnesium and calcium. The results showed that the recoveries from both serum and urine were very closely quantitative by direct titration (100%  $\pm$  0.8% S.D.). This does not necessarily mean that calcium and magnesium in the original materials are being quantitatively determined, since some may be intractably bound to some constituents of the sample. Further information was sought therefore in the second experiment, in which direct titrations of the untreated specimens were compared with those of wet-ashed material.

The results again indicated that within the limits of experimental error, quantitative measurement of both calcium and magnesium is achieved by direct titration. For urines, the ashing process appears to result in a slight loss of calcium and a greater loss of magnesium, the values for the former averaging 98.4%, and for the latter 93.5% of those for the original material. In any event, taking into account the results of all recovery experiments, it seems reasonable to assert that values obtained directly for urine, as well as for serum, are quantitative. This circumstance is fortunate in that it enables rapid determinations to be carried out with little or no preliminary preparation for titration. The methods have been found suitable for routine work, where time is normally a significant factor, as well as for occasional determinations.

**NORMAL VALUES** Sera from 19 male and 20 female blood donors, none of whom had made more than two previous donations, were assayed at least in duplicate for both calcium and magnesium. The age

of the men ranged from 18 to 56 years with a mean of 26 years, and of the women, 19 to 48 years with a mean of 30 years. The statistics are presented in Table I.

TABLE I  
NORMAL VALUES OF SERUM CALCIUM  
AND MAGNESIUM CONTENT FOR 20 FEMALE AND  
19 MALE BLOOD DONORS

Males			Females		
Age (yr.)	Calcium (mg.%)	Magnesium (mg.%)	Age (yr.)	Calcium (mg.%)	Magnesium (mg.%)
—	—	—	48	10.30	1.90
41	9.35	1.76	19	10.15	1.85
20	10.65	1.11	20	9.50	1.85
28	10.00	1.48	35	9.20	1.94
21	10.00	1.85	23	9.35	1.85
27	9.95	1.98	35	9.15	1.80
19	10.30	1.85	24	9.45	1.62
29	10.15	1.76	40	8.80	1.97
32	9.60	1.99	40	9.70	1.99
22	9.85	1.85	22	9.70	2.09
20	10.60	2.40	31	9.35	1.85
19	10.40	1.98	19	9.85	1.81
18	10.00	1.57	19	9.50	1.85
21	10.40	1.61	41	9.80	1.76
38	10.00	1.80	19	9.70	1.57
19	10.15	1.94	19	10.00	1.94
21	9.70	1.48	41	9.80	2.22
20	10.60	1.89	21	9.80	1.43
56	9.90	1.67	20	9.80	1.91
24	10.30	1.76	25	9.70	1.85
Means	10.10	1.78	Means	9.63	1.85
$\sigma$	$\pm 0.34$	$\pm 0.27$	$\sigma$	$\pm 0.35$	$\pm 0.17$
95% ranges	9.4-10.8	1.25-2.30	95% ranges	8.95-10.3	1.50-2.20

The following points of interest emerge. The mean serum calcium level for men is definitely higher than for women, but the spread of values as measured by the standard deviations is about the same. The mean serum magnesium levels, on the other hand, are almost the same for both sexes, but the range of values is distinctly smaller for women. No other information could be found concerning serum calcium and magnesium values for the separate sexes. For magnesium our overall mean of 1.82 mg. % (1.50 mEq./l.) may be compared with a mean of 1.37 mEq./l. for whole sera, or 1.43 mEq./l. for the corresponding ashed sera, calculated from the data of Zak *et al.* (1956). These workers made duplicate analyses on five pooled sera of unstated origin. The method employed was E.D.T.A. titration using murexide and eriochrome black T indicators. By similar methods, Carr and Frank (1956) obtained a mean value of 1.72 mEq./l. for a 'standard pool of serum'. Using flame photometry and plasma, Alcock, MacIntyre, and Radde (1960) found a mean of 1.66 mEq./l. for 76 adults; van Fossan, Baird, and Tekell (1959) a mean of 1.67 mEq./l.; and

Fawcett and Wynn (1961) a mean of 1.74 mEq./l. for 10 healthy subjects.

A summary of a number of results for plasma may be found in a study by Hanna (1961). In some of these the origin of the specimens is not stated. Montgomery (1961), using sera from 46 normal West Indian adults and employing flame spectrophotometry, obtained a mean of 1.70 mEq./l.; the primary standards in this case were made from magnesium sulphate. Dawson and Heaton (1961), in a thorough study using atomic absorption spectrophotometry, obtained a mean content of 1.84 mg. % (1.51 mEq./l.), in very close accord with our values for serum (Table I). The origin of the specimens is not stated; they are referred to as serum in the body of the text, but as plasma in their Table 6 of values. Assessment of all these results is difficult because of the different conditions prevailing in each case, but there does appear to be a tendency towards the finding of higher values by flame photometric procedures than by E.D.T.A. titration. The method of blood collection which we adopted may result in lower values, and it would certainly be of interest for future work to collect samples of blood at intervals during a complete donation. However, in a previous study of the serum iron content of normal donors (Beale, Bostrom, and Taylor, 1962), in which specimens were collected in the same way as at present, no significant difference was found between our means and those from other work. This suggests that no marked variation is to be expected in the calcium and magnesium content of blood taken at the beginning and end of donation. If this is so, the differences in reported magnesium levels may lie in the techniques used. Some values in the literature are undoubtedly high because of the use of impure magnesium oxide as a standard substance (see Hanna's article), and possibly because some magnesium salts are hygroscopic. The most suitable material is, in our experience, magnesium turnings of good quality. On the other hand calcium standards of high reliability may be prepared from the carbonate (provided this is heated at 200°C. for a number of hours) and the magnesium may then be checked against them. This procedure was adopted by Alcock *et al.* (1960) and in the present work. Nevertheless, these authors still obtained a higher mean value for plasma magnesium content than we

did for serum. A careful comparison of assays by flame and titration methods is needed, in order to decide whether or not a discrepancy exists between them.

The standard errors of measurement (S.E.M.) for the methods described were calculated by means of the relationships:

$$(\text{S.E.M.})^2 = \frac{\sum \Delta^2}{2N}$$

where  $\Delta$  = half the percentage difference between duplicates

$N$  = number of pairs of duplicates

$$\text{and } E_{\text{Mg}} = \sqrt{E_{\text{Ca+Mg}}^2 + E_{\text{Ca}}^2}$$

where  $E_{\text{Mg}}$  = S.E.M. for the magnesium determination

$E_{\text{Ca}}$  = S.E.M. for the calcium determination

$E_{\text{Ca+Mg}}$  = S.E.M. for the total ion determination.

This last relationship is applied because the magnesium determination involves the difference of two quantities each with their own error. For the reagents described under 'Methodology', we found the following values for the S.E.M. in each case:

$$E_{\text{Ca}} = \pm 0.5\%; \quad E_{\text{Ca+Mg}} = \pm 0.3\%; \quad E_{\text{Mg}} = \pm 0.6\%$$

This gives confidence limits of  $\pm 1.5\%$  for calcium and  $1.8\%$  for magnesium determination.

Approval for the publication of this work has been given by the Director of State Health Services, New South Wales Department of Public Health.

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