CLINICAL RESEARCH

Clinical validation of dialysable calcium in relation to other methods of serum calcium measurement

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Abstract

Dialysable calcium (CaD) values were measured by a simple technique not interfered with by protein bound calcium and validation attempted by comparison with concentrations of ionised calcium (CaI) and clinical categorisation. CaD values were also compared with total calcium (CaT) and albumin adjusted calcium (CaA) concentrations. The normal ranges for CaD, CaT, CaA, and CaI were calculated from the results in healthy blood donors. In 50 normal subjects CaD was more highly correlated with CaI than CaT or CaA. The effects of lying down and of venous stasis in 10 normal subjects showed that CaD was slightly influenced by posture only, whereas CaT was noticeably affected by posture and venous stasis; CaA reduced but did not abolish these effects on CaT. The correlation coefficient of CaD and CaI in patients with chronic renal failure was 0.81. CaD was compared with CaT and CaA values in 293 consecutive hospital patients; discrepant results were obtained in 14.3% and 13.0% of cases respectively and there were some clinical grounds for accepting CaD as correct in 86% and 74% of these cases.

Measurement of CaD is a simple, reliable method for estimating accurately the calcium concentration free from biologically inactive protein bound calcium.

Introduction

Although total serum calcium estimations in routine clinical laboratories have reached a high degree of precision, it has been

known for 50 years that the calcium bound to albumin and globulin—that is, roughly 40% of the total—is biologically inactive.¹ A continuous flow method for measuring dialysable calcium (CaD) was first described in 1977.² In this method ionised calcium, accounting for about half of the total calcium, and complex bound calcium, accounting for about 10% of the total calcium, are measured. The complex bound calcium is mainly in association with bicarbonate³ and to a less extent citrate and lactate. Despite the technical simplicity of this method and the fact that it is not influenced by calcium bound to plasma proteins it has not received widespread interest. We describe our experience in measuring CaD in various clinical settings and relate it to the concurrent measurement of total calcium (CaT), albumin adjusted calcium (CaA), and ionised calcium (CaI). Also we try to validate the use of CaD, firstly, by relating it to CaI and, secondly, by relating it to the clinical state of the patient.

Methods

CaD concentration was measured by the method of Toffaletti and coworkers,^{4,5} except that the sample buffer was changed from tris(hydroxymethyl)aminomethane (TRIS) to imidazole (P T Flett, N D H Balazs, personal communication). In brief, the method is a modification of the Technicon AutoAnalyzer II method for CaT, which is based on the dialysis of calcium across a semi-permeable cuprophane membrane and measurement colorimetrically using cresolphthalein complexone. Instead of adding a low pH buffer to the sample to release all protein bound calcium and hence measuring CaT, a 0.8M imidazole donor buffer at pH 7.4 was added to the sample stream. This had the effect of buffering the patient's serum towards a normal pH and thereby stabilising the normal equilibrium between bound and free calcium at that pH. Further to stabilise this equilibrium and reduce dialysis effects the donor buffer contained calcium carbonate 1.4 mmol/l (5.6 mg/100 ml). An AutoAnalyzer II manifold incorporating a 15 cm dialysis membrane was used. The recipient buffer was 0.025M imidazole pH 7.4. The chromagen cresolphthalein complexone was mixed with amino-methylpropanol buffer to prevent the pumping and mixing artefacts experienced when these were pumped separately and mixed on line. The chromagen used was shown to produce linear results up to a calcium concentration of 3.0 mmol/l (12.0 mg/100 ml). A primary 1.4 mmol/l (5.6 mg/100 ml) calcium carbonate standard (National Bureau of Standards, USA) was used and secondary calcium standards

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of 1.31 and 1.57 mmol/l (5.24 and 6.28 mg/100 ml) prepared from pooled patients' serum; these were used on a day to day basis. The between run coefficient of variation was 0.7% for the 1.31 mmol/l standard and 1.1% for the 1.57 mmol/l standard. Serum samples were collected from subjects without any special precautions and 500 μ l placed in the autoanalyser cups to accommodate the aspiration volume of 400 μ l.

CaT was assayed on a Corning 940 calcium titrator, in which the calcium in the sample complexed with fluorescein complexone to produce a green fluorescence. This was automatically titrated back to a non-fluorescent end point using ethylene glycol tetra-acetic acid. The volume of ethylene glycol tetra-acetic acid used was proportional to the calcium concentration. The interassay coefficient of variation at 1.94 mmol/l (7.76 mg/100 ml) was 1.4% and at 2.83 mmol/l (11.32 mg/100 ml) 1.3%. Albumin assays were performed on a Cobas-Bio Analyser using a bromocresol green succinate buffer reagent with the reading taken at 15 seconds. The interassay coefficient of variation was 2.5% at 28 g/l and 2.1% at 45 g/l. CaA was calculated from the formula $\text{CaA} = \text{CaT} + (40 - \text{albumin value in g}) \times 0.02$.⁶ CaI was measured on a Nova 2 calcium specific electrode. Samples for this procedure were collected anaerobically, stored at 4°C, and analysed within four hours. In this method no pH correction was employed, so that the CaI concentration was assumed to be measured close to the pH of the patient's serum.

Analysis of clinical categorisation was by review of the case notes of discordant cases. Correlation coefficients were calculated by the method of least squares analysis. Statistical evaluation of results was by paired or unpaired Student's *t* test, as appropriate.

Results

Figure 1 gives the results of CaD, CaT, and CaA estimations in 263 healthy blood donors (135 men, 128 women) aged 18 to 65. Figure 2 gives the results of CaI estimations in a separate group of 120 blood donors (60 men) aged 18 to 65. The normal range for each type of calcium measurement was defined as the mean and two standard deviations. The variance of the normal range of CaD was much smaller than for CaT or CaA but similar to that for CaI. Furthermore,

FIG 1—Distribution of concentrations of dialysable calcium (CaD), total calcium (CaT), and adjusted calcium (CaA) in healthy group of 135 men and 128 women blood donors.

Conversion: SI to traditional units—Calcium: 1 mmol/l $\approx$ 4 mg/100 ml.

FIG 2—Distribution of concentrations of ionised calcium (CaI) in healthy group of 60 men and 60 women blood donors.

Conversion: SI to traditional units—Calcium: 1 mmol/l $\approx$ 4 mg/100 ml.

Mean and SD (in parentheses) of normal ranges for men and women blood donors

	Men	Women
Dialysable calcium (CaD; mmol/l)	1.47 (0.05)	1.46 (0.06)
Total calcium (CaT; mmol/l)	2.34 (0.09)	2.29 (0.08)*
Albumin adjusted calcium (CaA; mmol/l)	2.28 (0.08)	2.22 (0.08)*
Ionised calcium (CaI; mmol/l)	1.19 (0.04)	1.19 (0.04)

*Difference from value in men $p < 0.001$.

Conversion: SI to traditional units—Calcium: 1 mmol/l $\approx$ 4 mg/100 ml.

FIG 3—Correlation of ionised calcium (CaI) and dialysable calcium (CaD) concentrations in 50 healthy volunteers.

Conversion: SI to traditional units—Calcium: 1 mmol/l $\approx$ 4 mg/100 ml.

there was no significant difference in the normal range for CaD or CaI between men and women, whereas the normal ranges for CaT and CaA were significantly higher for men than for women (table).

A further study of healthy volunteers aged 20 to 69 was made in which CaI was measured concurrently with CaD, CaT, and CaA. There were 50 subjects in all (13 women, 37 men), and figure 3 shows the correlation between CaI and CaD values. The correlation coefficient was 0.71, whereas the correlations between CaI and CaT and CaA in the same subjects were 0.48 and 0.41, respectively.

Figure 4 shows the effects of lying down and of venous stasis produced by inflating a sphygmomanometer cuff to halfway between systolic and diastolic blood pressure in five healthy men and five healthy women. In this study CaI was not measured. As expected, the changes in CaT concentration closely followed those for albumin. CaA showed a small effect of posture and venous stasis, while CaD was slightly affected by posture only.

The correlation between CaI and CaD was studied in 23 patients receiving haemodialysis for chronic renal failure. The correlation

coefficient of CaI and CaD was 0.89, similar to the correlation coefficients of CaI and CaT and of CaI and CaA (0.89 and 0.87, respectively).

The validity of CaD was compared with CaT and CaA in 293 consecutive hospital patients. The correlation coefficient between CaD and CaT was 0.86 (see fig 5). The normal ranges, derived from the blood donor population for CaD and CaT, are shown in figure 5, as are areas of discrepancy between CaD and CaT in which the value of CaD or CaT was low or high compared with a normal value of the other.

Area 1—Eighteen patients had normal CaD and high CaT values. In 13 cases the high CaT value was associated with a high albumin concentration and corrected into the normal range with CaA. A

lower in the hospital patients (many of whom were supine) than in the healthy ambulant blood donors. Furthermore, using the normal range derived from the CaT normal range for CaA is a widespread practice. The areas of discrepancy between CaD and CaA values are indicated in figure 6.

Area 1—Eight patients had normal CaD and high CaA values. In three patients the CaT concentration was normal, and in these there was no reason to suspect an abnormality of calcium metabolism. Two other patients had myeloma with raised immunoglobulin values. The remaining three were cases of apnoea in a neonate, Paget's disease, and inflammatory arthropathy.

Area 2—Of 12 patients with high CaD and normal CaA values, three had recurrent renal calculi and may have had primary hyperpara-

FIG 4—Effects of lying down and of venous stasis on serum albumin, total calcium (CaT), adjusted calcium (CaA), and dialysable calcium (CaD) values in five healthy women and five healthy men volunteers. Results are means and SEM.

* $p < 0.005$, ** $p < 0.01$ compared with value at zero time.

† $p < 0.05$, ‡ $p < 0.01$ compared with value at 60 minutes.

Conversion: SI to traditional units—Calcium: 1 mmol/l $\approx$ 4 mg/100 ml.

further three patients were hyperglobulinaemic (two with myeloma, one with inflammatory arthropathy), and the remaining two were cases of Paget's disease and apnoea in a neonate.

Area 2—Of seven patients with high CaD and normal CaT values, two had multiple renal calculi and renal calcification, and a further patient subsequently underwent parathyroidectomy for hyperparathyroidism. Although mild primary hyperparathyroidism was not proved in all of these cases, clinically it was considered possible.

Area 3—All 11 patients with normal CaD and low CaT concentrations were hypoalbuminaemic and corrected into the normal range for CaA.

Area 4—Of six patients with low CaD and normal CaT values, four had a clinical cause for the hypocalcaemia (one case each of pancreatitis, chronic renal failure, post-thyroidectomy state, recovery phase after cardiac arrest).

In 29 (9.9%) of the 293 cases, therefore, the CaT value was high or low when the CaD concentration was normal. Probably all these patients were normocalcaemic clinically. In 13 cases (4.4%) CaD was high or low when CaT was normal. In seven of these (54%) there were clinical grounds for suspecting a disorder of calcium metabolism.

The correlation coefficient between CaD and CaA values was 0.83 (see fig 6). The normal range for CaA used was actually the normal range for CaT as derived from the blood donor population. This was used because it reduced from 43 cases to 38 the discrepancy between CaD and CaA in this patient population when compared with the normal range actually derived from the blood donor population. This was largely because the mean serum albumin concentration was

thyroidism. One patient subsequently underwent parathyroidectomy for hyperparathyroidism, and a further patient had calcium gluconate gel applied to the skin for hydrogen fluoride burns. In seven cases there was no obvious cause for hypercalcaemia.

Area 3—Three patients had normal CaD and low CaA values. In all three the CaT concentration was normal and there was no reason to suspect an abnormality of calcium metabolism.

Area 4—Fifteen patients had low CaD and normal CaA concentrations. Of these, four had chronic renal failure, and two subsequently died; three had pancreatitis; and five were severely ill with various conditions. These 12 patients therefore had clinical conditions previously associated with a low CaI value.⁷ Acidosis, however, could not be excluded in all patients. In three cases there was no obvious cause for hypocalcaemia.

In 11 cases, therefore, CaA concentration was high or low when the CaD value was normal. Probably on clinical grounds all the patients were normocalcaemic. Of the 12 patients in whom the CaA value was normal when the CaD concentration was high, probably five (42%) were indeed hypercalcaemic. Of the 15 patients in whom the CaA value was normal when the CaD value was low, probably 12 (80%) were hypocalcaemic.

Discussion

The best technique for measuring the physiologically active calcium concentration has been a subject of controversy for

several years. Although the ionised fraction of calcium has been considered to be the physiologically active moiety,¹ most laboratories report CaT concentrations because the techniques of measurement are straightforward and reliable. In recent years a technique for measuring CaD has been developed,^{2-4,5} which in our hands is highly correlated with the measurement of CaI but overcomes many of the problems inherent in that technique. In validation of CaD as a measure of physiologically active calcium in normal subjects the correlation coefficient between CaD and CaI was appreciably higher than the

Fig 5

Fig 6

FIG 5—Correlation of dialysable calcium (CaD) with total calcium (CaT) values in 293 consecutive patients. Normal ranges indicated. Area 1: Normal CaD, high CaT. Area 2: High CaD, normal CaT. Area 3: Normal CaD, low CaT. Area 4: Low CaD, normal CaT.

Conversion: SI to traditional units—Calcium: 1 mmol/l $\approx$ 4 mg/100 ml.

FIG 6—Correlation of dialysable calcium (CaD) with adjusted calcium (CaA) values in 293 consecutive hospital patients. Normal ranges indicated. Area 1: Normal CaD, high CaA. Area 2: High CaD, normal CaA. Area 3: Normal CaD, low CaA. Area 4: Low CaD, normal CaA.

Conversion: SI to traditional units—Calcium: 1 mmol/l $\approx$ 4 mg/100 ml.

correlation coefficient between both CaT and CaI and CaA and CaI. Indeed, the calculation of CaA in these normal subjects resulted in a worse correlation of CaA with CaI than the correlation of CaT with CaI. If it is accepted that the CaI value is currently the best measure of physiologically active calcium, although influenced by the protein concentration to a small extent,⁸ then CaD provides a better measure than CaT or CaA. Measurement of CaD abolished the difference in the normal range between men and women, as did CaI but not CaT or CaA. In patients with chronic renal failure, in whom

the CaI value calculated from CaT and albumin has a poor correlation with the directly measured CaI,⁹ CaD was highly correlated with CaI. In our hands, however, so were CaT and CaD.

Technical problems in measuring the CaI concentration were that the blood had to be collected anaerobically, had to be analysed shortly after collection, and each sample had to be analysed individually, which was time consuming. By contrast, CaD required no particular precautions for collection or storage, the samples were occasionally analysed weeks after collection, and samples could be batch analysed in a continuous flow fashion.

CaD was also compared with CaT. In normal subjects CaT was noticeably affected by posture, the main effect occurring within 30 minutes; it was also greatly affected by venous stasis after three minutes, both effects probably being the result of changes in albumin concentration. By contrast, the changes in CaD with posture and stasis were minimal. Correlation with clinical categorisation is conceptually the most important validation of a biochemical test. In the study of hospital patients, although CaT and CaD were highly correlated, there was disagreement whether patients should be categorised as normocalcaemic, hypocalcaemic, or hypercalcaemic in 14.3% of cases. There were clinical grounds for accepting CaD as probably correct in 86% of the cases in which there was discordance. Toffaletti has performed a similar clinical categorisation analysis.¹⁰ Thus CaD probably represented the clinical state more accurately than CaT. Nevertheless, it must be accepted that this retrospective clinical categorisation analysis would have been strengthened by other, more definitive diagnostic techniques—for example, parathyroid hormone estimation or parathyroid exploration in the patients with renal stones and marginal increases of calcium concentration.

CaD was compared to CaA values in several settings. Numerous formulas for adjusting for variation in the concentration of albumin have been devised, and we selected the simplest and therefore the most widely used.⁶ CaA largely but not completely overcame the effects of posture and venous stasis on CaT. It was not as well correlated with CaI as was CaD in normal subjects. In the study of hospital patients there was a high degree of correlation between CaD and CaA. Nevertheless, in 13% of cases there was disagreement over classification of patients into normocalcaemic, hypocalcaemic, and hypercalcaemic groups. There were clinical reasons for accepting the CaD value as correct in 74% of these discordant cases. Ladenson *et al* used 14 algorithms for calculating the corrected calcium concentration from a knowledge of total calcium, protein, albumin, and pH and compared these with the free calcium concentration measured by an ion specific electrode.¹¹ They concluded that none of the algorithms predicted adequately the free calcium concentration. The calculation of CaA required measurement of both CaT and albumin with the inevitable accumulation of precision errors, whereas CaD required only one measurement. Thus the resources required to measure CaD are less than those required for CaA.

There are several potential problems with CaD. The first is that CaD is measured at a fixed pH no matter what the subject's actual serum pH is. Clearly the vast majority of patients will have an *in vivo* serum pH very close to 7.4, so that in most cases this correction will have little or no effect. In some patients with extremely low or high pH, however, CaD will be relatively inaccurate. This effect was investigated by Toffaletti and Bowers, who showed that the measured concentration of dialysable calcium decreased by approximately 0.05 mmol/l (0.2 mg/100 ml) for each 0.1 unit increase in pH between 7.2 and 7.6.⁴ The major advantage of correcting to a fixed pH is that it allows the serum sample to be collected without any special precautions, and in our opinion this outweighs the potential inaccuracy in a small number of patients.

Interestingly, the pH effect might account for the difference in our reference range for CaD (1.37–1.56 mmol/l; 5.48–6.24 mg/100 ml) compared with that of Toffaletti and Kirvan

(1.26-1.42 mmol/l; 5.04-5.68 mg/100 ml).⁵ Those workers used a trometamol (TRIS) buffer which is highly temperature dependent, whereas this is not the case for the imidazole buffer that we employed. Their buffer was made up to pH 7.4 at 37°C but used at room temperature. This would produce a rise in pH to roughly 7.7 at 25°C, which would be associated with a fall of approximately 0.15 mmol/l (0.6 mg/100 ml) in the measurement of CaD. As the mean of their reference range was 0.125 mmol/l (0.5 mg/100 ml) less than ours this technical difference may account for the differences in the normal ranges. This underlines the critical importance of the pH of the sample at which CaD is measured.

Another problem is that currently there is no internationally recognised reference standard for CaD. Our approach has been to calibrate the assay system against a solution of calcium carbonate, but this is open to criticism as it does not allow for the Donnan effect of protein. Nevertheless, in view of the fact that large changes in serum albumin concentration induced by venous stasis had no effect on CaD, the Donnan effect would appear to have no practical relevance. Although the problem of standardisation may be relevant to interpretation of results among different laboratories, once a standard has been developed within a particular laboratory the results will be internally consistent. Furthermore, if this method is widely accepted it should be possible to develop an acceptable international standard.

In favour of the use of CaD rather than other methods of calcium estimation is that the method is a simple modification of a continuous flow total calcium technique using equipment that has been tried and tested over many years and is readily available. It correlates well with CaI but has none of the practical problems associated with this technique. Although under many circumstances CaA gives a clinically equivalent result, calculation

requires the measurement of both CaT and albumin. Now that it is 50 years since the physiological irrelevance of protein associated calcium was first commented on it is time to move away from measuring CaT. In busy biochemical laboratories CaD estimation is an efficient and cost effective answer.

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The role of endogenous opiates in thermal regulation of the body during exercise

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Abstract

Naloxone abolished the rise in body temperature seen after bicycle ergometer tests performed by 10 healthy men. This suggests that endogenous opiates play a part in thermal regulation during muscular exercise.

Introduction

The effect of opiates on core temperature has been the subject of many studies. Evidence shows that endogenous opiates, in

particular endorphins, have a role in the central control of body temperature.¹⁻⁴ Because body temperature rises during dynamic muscular exercise we were intrigued to know whether endogenous opiates play a part in this phenomenon. We therefore studied the effect of naloxone (an opiate antagonist) on the changes in body temperature induced by exercise.

Subjects, methods, and results

Ten healthy men aged 22-28 years, who regularly participated in various sporting activities, underwent a graded ergometer test on three occasions. They exercised on a bicycle ergometer with electric brakes (Ergometric systems 380B, Siemens Elema, Sweden), and on each occasion an identical protocol was used. The tests were performed in the same laboratory at constant room temperature and humidity, at the same time of day, and with an interval of at least four days between each test for each subject.

The first test served as a control. In the second and third tests either placebo (5 ml saline) or 2 mg naloxone (naloxone hydrochloride 0.4 g/l, Dupont de Nemours, Belgium) was administered intravenously five minutes before exercising. The order of the placebo and naloxone tests was randomised and double blind; five subjects received placebo first and five received naloxone first to minimise carryover effects. The temperature in the laboratory was kept constant at 20°C and the humidity at 60%. Sublingual temperature was

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