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Method Comparison for Calcium Determination by Flame Atomic Absorption Spectrophotometry in the Presence of Phosphate

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We investigated the suppressive effects of phosphate on calcium determinations with lanthanum–air/acetylene and potassium–nitrous oxide/acetylene methods, and we evaluated the ability of these methods to meet the suggested analytical goals for urine samples. The 20 g/L La–air/acetylene method was the most nearly accurate for predicting the actual calcium concentrations (*t*-test value = −0.042), followed by the 2 g/L K–nitrous oxide/acetylene method (*t*-test value = 0.450), 10 g/L La–air/acetylene (*t*-test value = −0.733), and finally 5 g/L La–air/acetylene (*t*-test value = −2.446). The dilution used significantly influences the apparent calcium concentration measured with the La–air/acetylene methods.

Calcium determinations in biological fluids by atomic absorption spectrophotometry (AAS) present a challenge to clinical chemistry laboratories, given the number of potentially confounding variables (Table 1). In the present study we have investigated one of these variables, phosphate.

Phosphate combines with calcium in the condensed phase, forming a less volatile compound. This compound decreases the concentration of free vapor-phase calcium atoms in the spectrometer's light beam, thus causing a less intense analyte signal (*1*). This suppression can be improved by two different methods. The first, the air/acetylene flame (2300 °C) with an added releasing agent such as lanthanum, increases the absorbance signal by binding the phosphate and leaving free calcium atoms (*2*). However, lanthanum must be added in a molar ratio of lanthanum to phosphate of approximately 5:1 to ensure a maximum recovery of calcium (*2*), and even this will not totally alleviate the suppression (*1*). Alternately, the nitrous oxide/acetylene flame (3080 °C) overcomes the phosphate interference found in lower-temperature flames by producing a high calcium dissociation (*3*), which is unaffected by phosphorus content as great as 100-fold that of calcium (*13*). This apparent benefit is neutralized by increased ionization of calcium at higher temperatures, which reduces analytical sensitivity. Addition of an easily ionized compound, such as potassium, will eliminate this ionization interference (*3, 4*).

Our intent in this study was twofold: first, to compare the suppressive effects of phosphate on calcium absorbances with both the lanthanum–air/acetylene and the potassium–nitrous oxide/acetylene methods and, second, to determine the ability of these methods to meet the suggested analytical goals for urine samples, i.e., a standard deviation <2% of the actual calcium concentration (*14*) (SD = 0.2 mmol/L for 10 mmol/L calcium standard).

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Table 1. Variables Influencing Calcium Determination by Atomic Absorption Spectrophotometry

	Reference no.
Interferences	
Phosphorus	*1–8*
Sulfur	*5–7*
PH_3 contamination of acetylene	*1, 9*
Others (Si, Al, Ti, Zr)	*10*
(Be, V)	*11*
Releasing agents	
Lanthanum	*1, 2, 6*
EDTA	*1, 3*
Strontium	*8*
De-ionizing agents (potassium)	*3, 4*
Solvent	*2, 5*
Fuel type (temperature)	*3–5, 7, 12*
Flame height	*1, 5, 7*
Other adjustments to be optimized	
Dilution — Aspiration rate	
Fuel ratio — Position of light beam	
Position of burner — Lamp current	

Materials and Methods

Apparatus. We used a Model AA-1275 atomic absorption spectrophotometer (Varian Associates, Palo Alto, CA) and made all dilutions with a Hamilton digital dilutor (Hamilton Co., Reno, NV).

Procedures. Calcium standards from 0 to 2 mmol/L were prepared from 25 mmol/L calcium standard (Fisher Scientific Co., Fair Lawn, NJ) with various amounts of phosphate (0 to 200 mmol/L), which corresponds to the phosphate content of feline urine (*15*). Nitric acid (1.0 mol/L) was added to give a final acid concentration of 0.1 mol/L in each standard, to prevent the precipitation of $Ca(H_2PO_4)_2$ and $CaHPO_4$. We then assayed the standards with methods A2, B2, C2, and D2 (see Table 2). The standards in the La-containing solvents were used with the air/acetylene flame, the K-containing solvent with nitrous oxide/acetylene. The instrument settings are summarized in Table 3.

Urine samples. Urine samples were acidified by mixing one volume of 6 mol/L HNO_3 with nine volumes of urine. The samples were then analyzed for calcium by using methods B1, B2, C1, C2, D1, and D2 (Table 2), with a separate standard curve prepared for each method (generated by assaying the calcium standards with no added phosphate).

Results and Discussion

The results of assaying calcium phosphate standards with methods A2, B2, C2, and D2 are shown in Figure 1. The 20 g/L La method was found to be the most nearly accurate when compared with the actual calcium concentration (*t*-test value of −0.042). This was followed by 2 g/L K (t = 0.450), 10 g/L La (−0.733), and finally 5 g/L La

Table 2. Method Definitions[a]

Method	Abbreviation
Solvent	
La, 5 g/L[b]	A
La, 10 g/L	B
La, 20 g/L	C
K, 2 g/L[c]	D
Dilution	
1:10	1
1:20	2
1:30	3
1:40	4
1:50	5

[a] Solvents are represented first in the method identification, followed by the dilution. For example, La (10 g/L) with a 1:20 dilution is represented as B2.
[b] Published method for calcium determinations (*16*).
[c] Published method for calcium determinations (*4, 17*).

Table 3. Instrument Settings for Determination of Calcium by Atomic Absorption Spectrophotometry

	Air/acetylene method	Nitrous oxide/ acetylene method
Burner	air/acetylene	nitrous oxide/ acetylene
Fuel flow (units)	2.2	8
(L/min)	2.5	9.1
Oxidant flow (units)	6	6
(L/min)	16.6	13.5
Aspiration rate (mL/min)	5	4.6
The following settings were constant for both methods:		
Wavelength, nm	422.7	
Stoichiometry	fuel lean	
Slit width, mm	0.5	
Lamp current, mA	5	
Hold time, s	3	
Expansion scale	1	
Gas supply pressure, kPa		
Air	420	
Acetylene	70	
Nitrous oxide	280	

(−2.446). Although the 2 g/L K method was not the most accurate, it was the only method to approach the suggested analytical goal of a standard deviation (calculated from the difference between observed and actual calcium concentrations) of <2% (*14*). With a standard deviation of the difference of 0.049 mmol/L for calcium standards between 0 and 2 mmol/L, the 2 g/L K method was the most precise of the four methods but had the tendency to overpredict the actual calcium concentration. Descriptive statistics for the above methods are given in Table 4.

We chose the 10 g/L La method to compare the slope of each calcium curve as a function of phosphate concentration (Figure 2). The relationship was a second-order polynomial rather than a linear one, illustrating a greater suppressive effect with increasing phosphate concentration; this contrasts with previous studies (*5*), in which a different atomic absorption method was used.

The 5 g/L La method had the greatest suppressive effect, the La/PO_4 ratio varying between 27.3 and 3.4 for each phosphate concentration (25 to 200 mmol/L). This is in contrast to results with both the 10 g/L La (La/PO_4 = 54.6 to 6.8) and 20 g/L La (La/PO_4 = 109.2 to 13.6) methods.

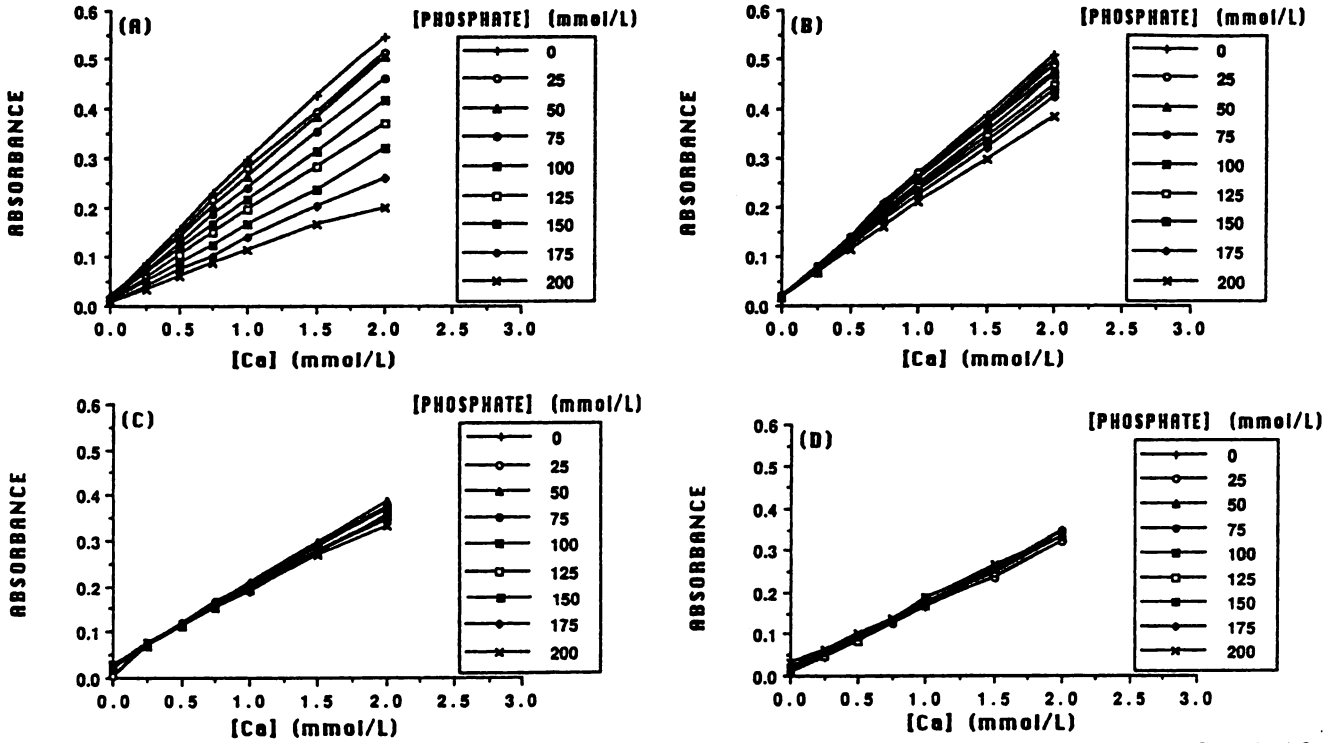

Fig. 1. Absorbance of calcium standards in the presence of various amounts of phosphate: (*A*) method A2, (*B*) method B2, (*C*) method C2, (*D*) method D2
See Table 2 for description of methods

Table 4. Effect of Various Methods on Calcium Concentration Measured

	Method			
	A2	B2	C2	D2
Overall mean, mmol/L[a]	0.596	0.775	0.852	0.910
Number of replicates per standard (63 standards)	4	4	4	4
Standard deviation, mmol/L	0.533	0.599	0.616	0.670
t-test value	−2.446	−0.733	−0.042	0.450
F-test value	5.984	0.537	0.002	0.202
P-value	0.0158	0.4651	0.9663	0.6538
Difference from actual, mean mmol/L	0.261	0.082	0.005	−0.053
SD	0.298	0.106	0.069	0.049

[a] Mean for all samples by all methods for the 63 standards was 0.857 mmol/L (SD = 0.658).

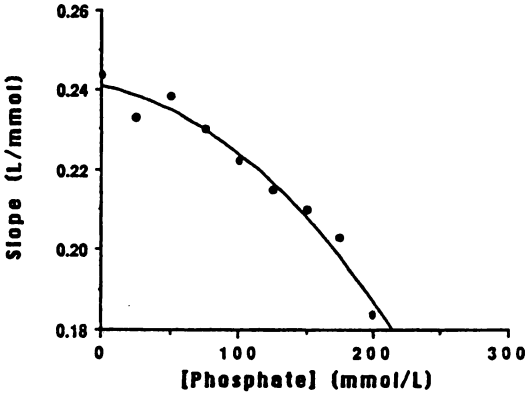

Fig. 2. Slopes from the B2 method plotted vs phosphate concentration

$y = 0.24 - (6.95 \times 10^{-5})x - (9.99 \times 10^{-7})x^2$ ($R^2 = 0.968$)

Because the La/PO_4 ratio influences the absorbance signal, we hypothesized that the dilution used would also be a variable within an individual La–air/acetylene method. To test this theory, we studied the 1 mmol/L calcium standards with varied phosphate concentrations (0 to 200 mmol/L), assaying these standards with methods B1–B5 and C1–C5. The results are shown in Figure 3. Although the absorbances were expected to decline with increasing dilutions, the variability within a dilution varied drastically. The 10-fold dilutions showed the greatest suppression in both the 10 g/L La and the 20 g/L La methods. The commonly used 10 g/L La (20-fold dilution) method also showed this suppression, whereas this effect was lessened in the 20 g/L La (20-fold dilution) method. The 30-, 40-, and 50-fold dilutions showed little variability in both the 10 and the 20 g/L La methods, which again illustrates the importance of the La/PO_4 ratio.

Calcium determination in urine samples is much more dynamic than the prepared calcium phosphate standards used above. For this reason, we compared six different methods (B1,B2,C1,C2,D1, and D2) by using 34 feline urine samples. Because the actual calcium concentrations were unknown, and because the C2 method had been found to be the most nearly accurate with the standards, we compared all observed calcium values with those by the C2 method (Table 5). The 2 g/L K (10-fold dilution) showed the least variation (SD = 0.08 mmol/L) followed by 2 g/L K (20-fold dilution) (SD = 0.087 mmol/L), 10 g/L La (20-fold dilution) (SD = 0.124 mmol/L), 20 g/L La (10-fold dilution) (SD = 0.124 mmol/L), and finally 10 g/L La (10-fold dilution) (SD = 0.177 mmol/L).

We found the 20 g/L La–air/acetylene method to be the preferred technique. However, methods involving La concentrations <20 g/L encounter serious phosphate suppression. Even at this La concentration and excessively high phosphate, the calcium signal will still be suppressed. Therefore, the greatest La/PO_4 ratio possible (without exceeding sensitivity limits) should be used to reduce this suppressive effect. We preferred the 2 g/L K–nitrous oxide/acetylene method over the low-La concentration techniques, but it has a number of drawbacks. First, it has the tendency to consistently overpredict the actual calcium concentration. Second, nitrous oxide is expensive, not readily available, and requires a special burner. Third, use of the optimal fuel setting is critical. A fuel-lean flame (fuel flow rate <6 L/min) has the greatest sensitivity, but carbon

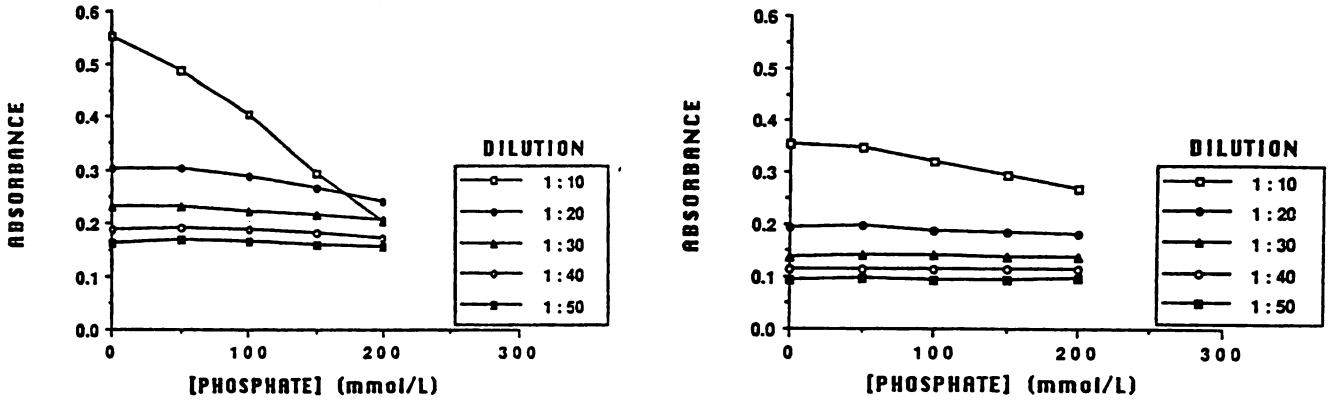

Fig. 3. Absorbance of 1 mmol/L calcium standard plus various amounts of phosphate in methods B1–B5 (*left*) and methods C1–C5 (*right*)

Table 5. Various Methods Compared with the C2 Method for 34 Feline Urine Samples

	Method				
	B2	D2	B1	C1	D1
Overall mean, mmol/L[a]	0.388	0.499	0.211	0.395	0.460
Number of replicates per urine sample	3	3	3	3	3
Standard deviation, mmol/L	0.274	0.305	0.243	0.266	0.283
t-test value	−0.795	0.715	−3.441	−0.719	0.198
F-test value	0.632	0.511	11.839	0.517	0.039
P value	0.4295	0.4771	0.001	0.4747	0.8438
Difference from mean C2 method, mmol/L	0.035	0.05	0.117	0.034	0.033
SD	0.124	0.087	0.177	0.124	0.08

[a] The overall mean for the C2 method was 0.445 mmol/L (SD = 0.316)

builds up on the burner, which progressively decreases the absorbance signal. A fuel-rich flame (fuel flow rate >10 L/min) drastically decreases the sensitivity and is accompanied by precipitation of potassium chloride in the nebulizer; this progressively decreases the sample-uptake rate, which also reduces the absorbance signal. These problems can be reduced by optimizing the fuel flow rate and running distilled water through the nebulizer for ~30 s between each sample.

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