A Method for the Analysis of Phosphate and Calcium in Small Samples of Plasma by Atomic Absorption Spectrophotometry

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Inorganic phosphate is determined by a molybdenum method by using atomic absorption spectrophotometry, on the basis of the observations of Berenblum & Chain (1938) and Zaugg & Knox (1966, 1967). The method permits analysis for calcium in the same sample and can detect $0.1 \mu\text{g}$ of P. The procedure given below is designed for automatic diluters and dispensers and uses 0.2 ml of plasma. It is suitable for phosphate concentrations between 0.5 and 20 mg of P/100 ml and calcium concentrations between 1 and 20 mg of Ca/100 ml. Analyses have a mean coefficient of variation of 1%. Since only one-quarter of the sample is needed after deproteinization, the sample volume could be reduced at the cost of a small loss in convenience and precision.

Fig. 1 shows the results of testing by the method

of additions. Successive increments of phosphate or of calcium added to samples of plasma increased the observed concentrations by the calculated amount, without mutual interference.

Principle. Excess of molybdate and a buffer are added to a deproteinized sample, followed by an immiscible solvent. In the optimum pH range (1.7-2.1) phosphomolybdic acid is quantitatively extracted. Free molybdic acid is then complexed by adding citrate. Phosphate is determined by spraying the solvent layer for molybdenum, and calcium by spraying the aqueous phase after removal of residual organic solvent.

Conditions investigated. Studies of the optimum conditions for the method are summarized as follows.

(a) Solvent. Extraction of phosphomolybdic acid from water requires an immiscible solvent that

Fig. 1. Phosphate and calcium measurements in plasma, tested by the method of additions. Successive increments of phosphate or calcium increased the observed concentrations by the calculated amount, without mutual interference. The lines drawn in are the theoretical lines of zero and unit slope.

is nonetheless relatively polar. Isobutyl methyl ketone proved suitable and is a good solvent for flame spectrophotometry (Allan, 1961). In a mildly reducing flame molybdenum dissolved in it absorbs intensely. Small increases in acetylene flow increase background absorption minimally without altering the slope of the calibration graph. The viscosity of isobutyl methyl ketone is low and variation in analyses due to this cause has a temperature coefficient of less than 0.5%/°C.

(b) Buffer. Even in the absence of phosphate, isobutyl methyl ketone begins to extract significant amounts of molybdic acid below pH 1.5. Extraction of phosphomolybdic acid becomes incomplete above pH 2.1. Samples deproteinized with perchloric acid or trichloroacetic acid therefore require partial neutralization and a buffer is needed to make the method convenient and reproducible.

Many of the buffers with suitable pK that were tested increased molybdenum extraction in the absence of phosphate (e.g. malonic acid) or formed precipitates with molybdate under the conditions of analysis (e.g. glycine, β -alanine). However, formic acid and α -alanine proved satisfactory. Solutions containing formate and molybdate keep poorly and α -alanine is the most convenient of the buffers found.

(c) Extraction. Extraction of phosphomolybdic acid from the aqueous phase is not instantaneous. It may involve displacement of an equilibrium in which molybdenum shows several valencies (Killeffer & Linz, 1952). Our studies (J. A. Parsons, B. Dawson, E. Callaghan & J. T. Potts, jun., unpublished work) indicate, surprisingly, that 15 atoms of Mo are extracted for each atom of P. As in the methods of Marsh (1959) and Zaugg & Knox (1966), citrate is added after the extraction and shaking repeated. This has the effect of complexing free molybdic acid and decreasing the blank virtually to zero.

Maximum precision and reproducibility are critically dependent on the adequacy of shaking and subsequent separation of the phases. All operations are carried out at room temperature and tubes should neither be heated with the hand nor exposed unnecessarily to the flame. A mechanical shaker was found convenient for batches of 50–100 samples. Small racks can be adequately shaken by hand, but insufficient vigour is the commonest cause of variation between replicates. Deproteinized plasma samples were found to need more vigorous shaking than standards made from aqueous phosphate solutions.

Centrifugation after the second shaking must be sufficient to produce perfect clearing. Large temperature differences should be avoided at this stage too, since the mutual solubility of isobutyl methyl ketone and water falls sharply with rising

temperature (Gross, Rintelen & Saylor, 1939) and heating may cause cloudiness.

Recommended procedure. The sample (0.2ml) is diluted with 1.8ml of distilled water. Perchloric acid reagent (1) (2.0ml) is added and the mixture centrifuged (at 2000g for 10min). A portion (1.0ml) of the deproteinized supernatant is transferred to a hard-glass tube with 2.5ml of distilled water, and molybdate–alanine reagent (2) is added (0.5ml). The mixture should have pH 1.9. This is checked when making new reagents and the volume of acid in reagent (2) adjusted if necessary. Isobutyl methyl ketone (3) is added (5ml), silicone rubber stoppers are inserted and tubes are shaken vigorously for 60s. Some silicone rubbers contain calcium, but Dow–Corning and Esco stoppers have proved satisfactory. Citrate reagent (4) (1.0ml) is added, stoppers are re-inserted and shaking is repeated (60s). After being centrifuged (at 2000g for 10min) to break the emulsion, samples are ready for spraying.

For both molybdenum and calcium, atomic absorption spectrophotometry is carried out in a mildly reducing (slightly luminous) flame. The following conditions are suitable for a Perkin–Elmer model 303 spectrophotometer with slit 4 and a Belling three-slot burner at an air pressure of 30lb/in².

For molybdenum, the flow gauges should read air 9, acetylene 7. In the absence of organic solvent this mixture would give a very blue flame, which might damage the burner, and isobutyl methyl ketone saturated with water should therefore be aspirated during warm-up (10min) and between samples. It is convenient to use the line at 313.3nm for sample concentrations up to about 6mg of P/100ml and the line at 390.3nm, which gives about threefold lower sensitivity, for higher values.

For calcium, flow gauges should read air 9, acetylene 9, and the burner should aspirate water between samples. The line at 422.7nm gives convenient absorptions at all calcium concentrations likely to be encountered in biological fluids.

The calibration curve of molybdenum extinction against phosphate concentration falls off slightly from linearity at the upper end of the recommended range, and a graph obtained with close standards is recommended for the most accurate results. For large numbers of analyses automatic readout devices are available. Alternatively, results can be read directly from a recorder chart by using a transparent reader, made empirically to accommodate the similar but not identical calibrations obtained on different days. Such a family of related scales is generated by projecting a calibration curve on to a vertical line and joining points on this by gently sloping lines to a distant origin.

Reagents. These are made up as follows.

(1) Perchloric acid (60%), 121ml, or (70%), 104ml, and distilled water to 1 litre.

(2) Sodium molybdate, 84.7g, DL- α -alanine, 89.1g, 5M-hydrochloric acid, 34.4ml, and distilled water to 1 litre. This reagent should be protected from exposure to sunlight.

(3) Isobutyl methyl ketone saturated with distilled water.

(4) Citric acid, 143g, aq. ammonia (sp.gr. 0.88), 56ml, and distilled water to 1 litre. This reagent should be kept refrigerated (up to 1 month).

(5) Phosphate standards. Solutions containing 0.05–2.0mg of P/100ml in distilled water are diluted with their own volume of reagent (1) and are then stable. Portions (1ml) of those in the expected range are subjected to the procedure as though they had been deproteinized and match 0.5–20mg of P/100ml in the original samples.

(6) Calcium standards. Solutions containing 0.8, 1.0 and 1.2mg of Ca/100ml in distilled water are diluted with their own volume of reagent (1) and are then stable. Portions (1ml) are subjected to the procedure as though they had been deproteinized and match 8, 10 and 12mg of Ca/100ml in the original samples.

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