Micro-method for the estimation of calcium by AutoAnalyser[1]

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The standard AutoAnalyser technique for the determination of calcium in plasma or other biological materials is based on the photometry of calcium murexide. Using this method we have been unable to obtain sufficient sensitivity to measure accurately the calcium content of plasma. Other disadvantages of this method include base-line drift due to instability of murexide, and considerable contamination from sample to sample. Moreover, the estimation involves the diffusion of calcium across a semi-permeable membrane, and the diffusion rate of calcium from an aqueous standard differs from that of a protein-containing solution such as plasma.

Wieme and van Raepenbusch (1962) described an AutoAnalyser method using plasmacorinth B instead of murexide, based on the manual method of Kingsley and Robnett (1957, 1958). We have increased the sensitivity of their method by eliminating the 37°C. water-bath, by using a more favourable wavelength, and by employing a 15 mm. flow cuvette. The plasmacorinth B concentration has been reduced to lower the blank value. These modifications permit the estimation of calcium concentration in 0·2 ml. of plasma. The method is also suitable for the estimation of calcium concentration in both urine and faeces. We have confirmed that interference by magnesium and phosphate is very small.

REAGENTS

All the distilled water used was further purified by deionization.

4% w/v SODIUM HYDROXIDE This solution contains 0·4% w/v AP 14 surface-active agent (I.C.I.).

PLASMACORINTH B STOCK SOLUTION (200 mg./100 ml.) Plasmacorinth B (G. Gurr), 500 mg., is dissolved in about 200 ml. of distilled water, containing 0·75 ml. of 0·1N hydrochloric acid, with vigorous stirring for about one hour, and then made up to 250 ml. with distilled water. Stored in a dark bottle at room temperature the solution is stable for at least three months.

PLASMACORINTH B WORKING SOLUTION (40 mg./100 ml.) Plasmacorinth B stock solution, 20 ml., is diluted to 100 ml. with distilled water with the subsequent addition of 0·5 ml. of 0·1N hydrochloric acid. Stored in a dark bottle at room temperature this solution is stable for one week. This quantity is sufficient for 90 minutes running time.

CALCIUM STANDARD STOCK SOLUTION (400 mg./100 ml.) We use a solution of Specpure calcium chloride, supplied ready made by Johnson, Matthey & Co. Ltd.[2]

CALCIUM STANDARD INTERMEDIATE STOCK SOLUTION (100 mg./100 ml.) Stock standard, 50 ml., plus 1·0 ml. of concentrated hydrochloric acid, diluted to 200 ml. with distilled water is stored in a refrigerator.

CALCIUM STANDARD WORKING SOLUTIONS Dilute 10, 15, 20, and 25 ml. volumes of intermediate stock solutions to 200 ml. with distilled water, to give working standards of 5, 7·5, 10, and 12·5 mg./100 ml. respectively. Store in a refrigerator.

PREPARATION OF SPECIMENS

BLOOD Heparinized plasma (10 units sodium heparin/ml. blood) or serum may be used.

URINE When the urine is received in the laboratory, a representative sample of 25 ml. is taken and 2 drops of glacial acetic acid (A.R.) added to give a final *p*H of approximately 3.

FAECES A timed collection of faeces is thoroughly homogenized with the addition of distilled water as necessary and then weighed. A sample corresponding to a one-hour collection is weighed into a 250 ml. conical flask and 25 ml. of concentrated nitric acid added. The flask is then boiled in a fume cupboard until the solution appears clear. After cooling add 25 ml. of distilled water and adjust the *p*H to approximately 5 by indicator paper, adding first 40% w/v sodium hydroxide solution very slowly, followed by N sodium hydroxide solution for the final adjustment. Filter through a Whatman no. 44 paper into a 100 ml. volumetric flask and make up to volume with distilled water.

DILUTION OF SPECIMENS

This is best carried out with two syringe pipettes of capacity 0·2 and 1·0 ml.

STANDARD AND PLASMA 0·2 ml. is added to 1·0 ml. of distilled water; final dilution 1/6.

URINE AND FAECES 0·2 ml. is added to 2·0 ml. of distilled water; final dilution 1/11.

If the reading is less than that of the 5·0 mg./100 ml. standard, the estimation is repeated using 0·4 ml. sample in the case of plasma (dilution 1/3·5) and 1 ml. of distilled water in the case of urine and faeces (dilution 1/6).

PROCEDURE

The manifold is illustrated in Figure 1. The colorimeter uses a pair of 623 mμ filters, and a density filter combined with the no. 1 aperture. This density filter was constructed from *x*-ray film of a sufficient density to allow a baseline setting of 23% transmission to be obtained with

[1]AutoAnalyser is the trade name of Technicon Instruments Co.

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[2]Johnson Matthey & Co. Ltd., 78 Hatton Garden, London, E.C.1

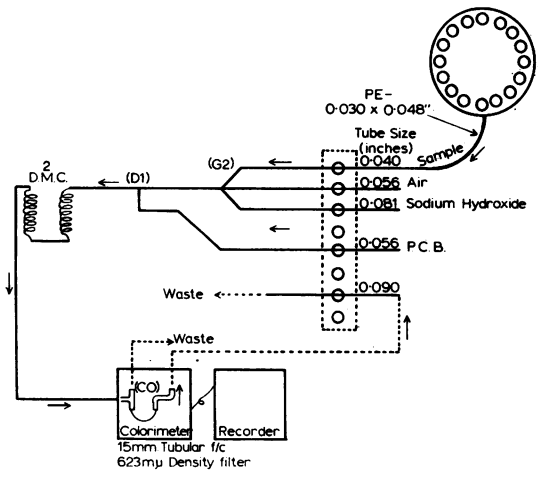

FIG. 1. *Flow diagram.*

the percentage transmission control at a setting between 8 and 9. The density filter can be permanently fixed to the no. 1 aperture. The wavelength of 623 mμ was chosen for the filters as this represented the maximum difference between plasmacorinth B and its calcium complex, as shown by their spectrophotometric absorption curves (Fig. 2). With the reagents flowing and with the sample lead in distilled water the baseline is set at 23% transmission, and this is the baseline used for the measurement of all the peaks. The baseline obtained when sampling air is lower than the 'true' baseline, due to the decrease in dilution of the colour reagent. The clearance between samples is good and the prompt and complete return to baseline is shown in Figure 3. The recorder is set to 'normal' and the samples are run at a rate of 40 per hour. The four calcium standard solutions are placed in the first and last four cups and a 10·0 mg./100 ml. standard sample after every six unknowns. The sensitivity should be such that the 12·5 mg./100 ml. calcium standard solution reads greater than 90% transmission; if necessary a variation in transmission can be obtained by small variations in baseline setting. On our machine a baseline setting of 23% transmission

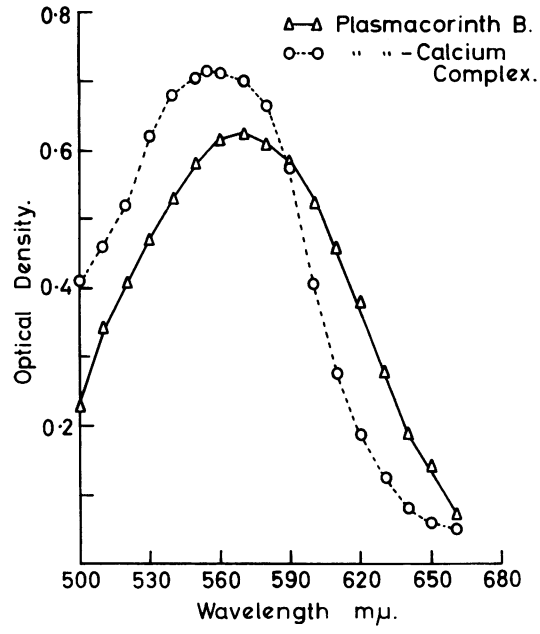

FIG. 2. *Spectrophotometric absorption curves of plasmacorinth B and its calcium complex.*

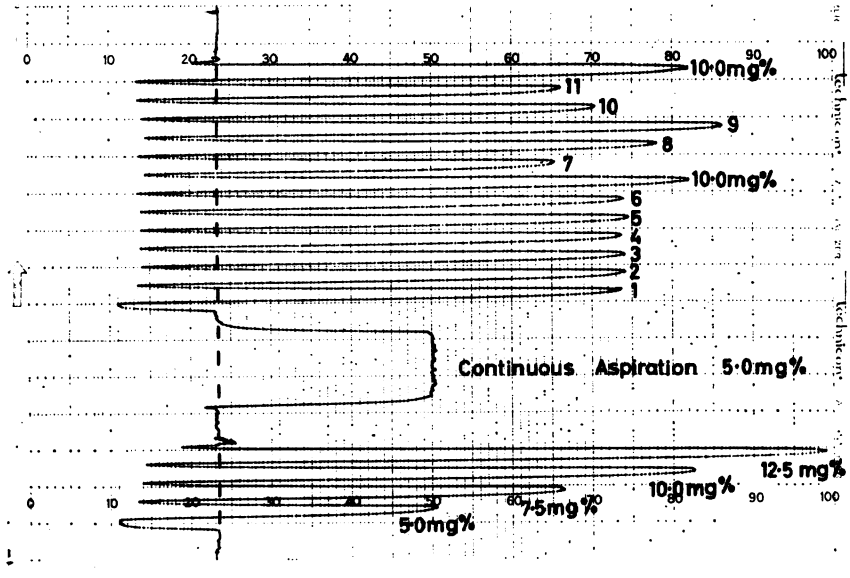

FIG. 3. *Unretouched photograph of recording showing calibration, continuous aspiration of a standard solution, reproducibility of pooled plasma samples (1-6), and typical plasma results (7-11).*

gives excellent sensitivity which is reproducible from day to day. Contamination from sample to sample in our system is negligible and it is therefore unnecessary to include water washes between samples.

RESULTS

The calibration curve is linear between 5·0 and 12·5 mg./100 ml. (Fig. 4). The sensitivity of the method permits the chart to be read to 0·1 mg./100 ml. (0·5% transmission). The reproducibility of plasma samples and standards is within 0·2 mg. calcium/100 ml. and the recovery of calcium added to plasma, urine, and faeces is between 92 and 104% (Table I). Comparison of the results obtained on 24 plasma samples (range 7·5 to 11·0 mg./100 ml.) and 20 urine samples (range 5·0 to 30·0 mg./100 ml.) with this method and with the E.D.T.A. titration method of Fales (1953) has shown differences within the range of ± 0·2 mg./100 ml. for plasma specimens and ± 0·5 mg./100 ml. for urine specimens. The mean values for the specimens tested by this method were 9·40 mg./100 ml. for plasma and 10·40 mg./100 ml.

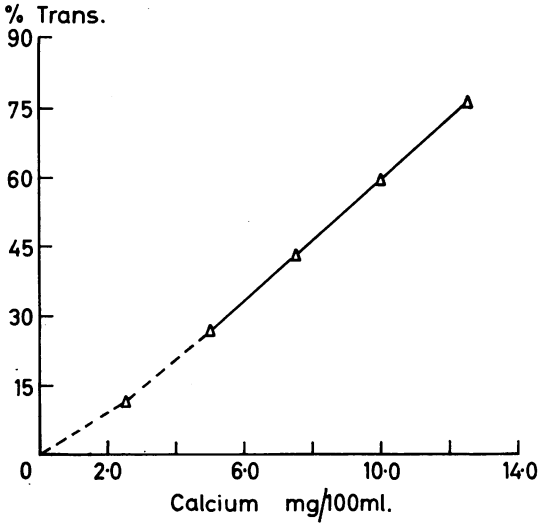

FIG. 4. *Calibration curve.*

TABLE I

RESULTS

Specimen	*Original Calcium (mg./100 ml.)*	*Calcium Added (mg./100 ml.)*	*Estimated Calcium (mg./100 ml.)*	*Recovery*	
				Actual (mg./100 ml.)	*Recovery (%)*
Plasma	9·50, 9·40, 9·40, 9·40, 9·50, 9·50 (9·45)	2·00	11·50, 11·50, 11·40, 11·40, 11·50, 11·40 (11·45)	2·00	100
	ditto	5·00	14·10, 14·10, 14·10, 14·30, 14·10, 14·30 (14·15)	4·70	94
Urine	13·80	16·00	29·00	15·20	94
	6·90	16·00	21·50	14·60	92
	1·40	16·00	17·40	16·00	100
	1·40	8·00	9·40	9·00	100
Faeces	10·90	10·80	22·10	11·20	104

for urine as compared with 9·57 mg./100 ml. and 10·10 mg./100 ml. respectively using the method of Fales.

The possible interference of magnesium, phosphate, and bilirubin was investigated by substituting a solution of magnesium chloride, potassium dihydrogen phosphate, and bilirubin in 0·1N sodium hydroxide for the distilled water diluent. An increase in magnesium equivalent to 5·0 mg./100 ml. of plasma increased the apparent calcium level by less than 0·2 mg./100 ml. An increase of phosphate concentration by the equivalent of 10·0 mg./100 ml. of plasma decreased the apparent calcium level by less than 0·2 mg./100 ml. Bilirubin levels equivalent to 5·0 mg./100 ml. of plasma produced an apparent decrease of the calcium level of 0·2 mg./100 ml. and a level equivalent to 35·0 mg./100 ml. plasma an apparent decrease of 0·5 mg. calcium/100 ml.

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REFERENCES

Fales, F. W. (1953). *J. biol. Chem.*, **204**, 577.
Kingsley, G. R., and Robnett, O. (1957). *Amer. J. clin. Path.*, **27**, 223.
——, —— (1958). *Ibid.*, **29**, 171.
Wieme, R. J., and van Raepenbusch, F. R. (1962). *Clin. chim. Acta*, **7**, 883.