An Application of Yoshimatsu's Blood¹⁾ Calcium Method to Human Milk.*

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The present paper treats of an application of Yoshimatsu's method for blood calcium to human milk. I found that if Yoshimatsu's blood calcium method is directly applied to human milk, the result is sometimes unsatisfactory. The reason is, it seems to me, partly due to an insufficient deproteinisation, and partly to a much larger fluctuation of calcium content in human milk than in blood. Below I shall describe those procedures necessary for accurate determination of calcium in human milk.

Reagents used.

Here I shall mention all the reagents necessary for the determination of human milk calcium; most of these are of course the same used in Yoshimatsu's original method.

1. Stock standard solution.

0.4100 grms. of calcium-oxyquinoline complex, prepared as described below, are dissolved in 50 c.c. of normal HCl solution and then made up to exactly 500 c.c.

Preparation of calcium-oxyquinoline complex.

To 2.5 liters of distilled water put about 5 grms. of Seignette salt in 100 c.c. of 1% pure calcium chloride solution, then add 25 c.c. of

1) Sh. Yoshimatsu, Tohoku J. Exp. Med., 1930, 15, 355.

* 22nd Report on Micro Method.

normal sodium hydroxyde solution and mix. Add a few c.c. of 5% alcoholic orthoxyquinoline solution and mix well. Wait half an hour, then collect the calcium-oxyquinoline precipitates on a Buchner funnel and wash several times with 5% alkaline Seignette salt solution (8). Dry at 120°C.

2. Standard solutions.

a. Pipette exactly 25 c.c. of the stock solution (1) into a 500 c.c. volumetric flask and make up to the mark with distilled water. 2 c.c. of this solution is equal to 0.01 mgrm. of calcium.

b. 37.5 c.c. of the stock solution (1) are diluted with distilled water and made up to 500 c.c. 2 c.c. of this solution are equal to 0.015 mgrms. of calcium.

Note to the standard solutions:—

The fluctuation of calcium content in human milk is so wide, that it is necessary to use, besides the standard solution (2 a) in the original, the above mentioned Reagent (2 b).

3. 5% alcoholic o-oxyquinoline solution.

Dissolve 5 grms. of o-oxyquinoline in 100 c.c. of 95% ethyl alcohol.

4. 33% Seignette salt solution.

Dissolve 50 grms. of sodium potassium tartarate in 100 c.c. of distilled water.

5. Aqua ammoniae (sp. w. 0.96).

6. Normal NaOH solution.

7. Normal HCl solution.

8. 5% alkaline Seignette salt solution.

Dissolve 5 grms. of Seignette salt in 100 c.c. of distilled water and add 20 c.c. of normal NaOH solution (6) to it.

9. Ammoniacal ammonium chloride solution.

Add 34 c.c. of aq. ammoniae (5) to 1,000 c.c. of 5% ammonium chloride solution.

10. 0.01 N. HCl solution.

11. Solution of sodium sulphate and sodium tungstate.

15 grms. of sodium sulphate anhydride and 7 grms. of sodium tungstate are dissolved in distilled water and made up to 1,000 c.c.

12. $\frac{1}{3}$ N sulphuric acid solution.

13. Phenol reagent.

Transfer to a flask having the capacity of about 1,500 c.c., 750 c.c. of distilled water, 100 grms. of sodium tungstate, 20 grms. of phosphomolybdic acid, 50 c.c. of 85% phosphoric acid. Partly close

the opening of the flask with a watch glass, and boil the mixture gently for two hours. Dilute to one liter with distilled water.

14. 20% sodium carbonate solution.

25 grms. of sodium carbonate ($\text{Na}_2\text{CO}_3 + 10\text{H}_2\text{O}$) is dissolved in 100 c.c. of distilled water.

Method used.

1. Deproteinisation of human milk:—

To 4 c.c. of solution of sodium sulphate anhydride and sodium tungstate (11) in a 10 c.c. reagent glass, add exactly 0.5 c.c. of human milk and mix. After 5 minutes add 0.5 c.c. of $\frac{1}{2}$ N sulphuric acid (12) and mix thoroughly. Wait 30 minutes and filter through filter paper (Toyo filter paper No. 3).

2. Proper procedure:—

Pipette 0.5 c.c. of the milk filtrate, obtained in the way described above, into a 5 c.c. conical centrifuge tube* and add 0.5 c.c. of distilled water, 0.15 c.c. of 33% Seignette salt solution (4) and 0.25 c.c. of normal NaOH solution (6) and mix. To this 0.15 c.c. of 5% alcoholic o-oxyquinoline solution is added drop by drop as in the original and mix thoroughly. By stirring the mixture it is much better to continue stirring the mixture for 3 minutes instead of for 2 minutes as in the original. However,

Fig. 1.

the inside of the tube must not be rubbed, or the result obtained may be too high on account of a possible contamination of calcium of glass. Then dip into hot water bath (Ca. 95°C) instead of boiling water, otherwise the contents may abruptly boil over as was remarked by M. Yoshida,²⁾ and let it remain there for about 2 minutes. Cool at room temperature and wait 30 minutes or longer, instead of 15 minutes as in the original, stirring it occasionally. Then centrifuge sharply, syphon out the supernatant fluid completely with hooked narrow glass tube (Fig. 1).

Add 1 c.c. of alkaline Seignette solution (8) into the tube and wash the precipitate, mixing with glass rod. Centrifuge and syphon out

* The point of it must be narrow enough to collect the calcium-oxyquinoline precipitate formed but not too narrow, lest the point of stirring glass rod should easily break in it.

2) M. Yoshida, Tohoku J. Exp. Med., 1934, 22, 468.

again. Then add 1.5 c.c. of Reagent (8) washing the inside of the tube. Centrifuge at once (not mixing the precipitate this time) and syphon out completely. Now washing is sufficient; do not wash so often as four times as described in the original, for the precipitate may be washed away as M. Yoshida noticed. Then add one c.c. of Reagent (9) and stir and place the tube in a boiling water bath. Wait one minute, then add one drop of Reagent (5) and mix. After three minutes add again one drop of Reagent (5). Thus after 5 minutes from the beginning, filter while hot through a Jena glass micro filter 12, G. 3 with an asbestos sheet with suction into a 10 c.c. volumetric flask (Fig. 2). Wash the precipitate three times with 0.5 c.c. of hot distilled water, and collect all the filtrate and washing water in a 10 c.c. volumetric flask, and then add 0.5 c.c. of 0.01 N HCl(10). Into two other flasks of 10 c.c. volume, pipette exactly 2 c.c. of Reagent (2 a) and Reagent (2 b) respectively, one drop of Reagent (5) and 1.5 c.c. of distilled water. Add to these three flasks 1.2 c.c. of Reagent (14) and one c.c. of Reagent (13). Place the flasks in a boiling water bath for 5 minutes, then cool at the room temperature. Make up each flask with distilled water to the mark and mix. Compare the colours thus obtained with the micro colourimeter type Duboscq, setting the standard at 30 m.m. in the case of Reagent (2 a) and at 15 m.m. in the case of Reagent (2 b).

Fig. 2.

$$\frac{\text{Reading of standard [Reagent (2 a)]}}{\text{Reading of unknown}} \times 0.01 \times 2 \times 1,000 \text{ mgrms. Ca per 100 c.c.}$$

$$\frac{\text{Reading of standard [Reagent (2 b)]}}{\text{Reading of unknown}} \times 0.015 \times 2 \times 1,000 \text{ mgrms. Ca per 100 c.c.}$$

Precautions about the filtration through a Jena glass micro filter:—

The fluid must not become cold; and distilled water for washing the precipitate must also be kept always hot. The fluid and washing water must be poured into the filter very cautiously along a glass rod, lest the asbestos below should rise to the surface of the fluid now in the filter. The suction must be mild enough to permit the filtrate to fall only drop by drop, otherwise it may splash out from the flask.

Only at the end of the filtration may the suction be made stronger so as to make the filtration complete.

Discussion.

The deviation of the results obtained by this modification from the theoretical values will be given in Table 1 (Cf. Table 1).

The recovery of calcium added to human milk is shown in Table 2 (Cf. Table 2).

TABLE 1.
Analysis of pure calcium solution.

Theoretical value, mgrms.	Found mgrms.	Error %	Theoretical value, mgrms.	Found mgrms.	Error %
10.5	10.7	+2	10.5	10.7	+2
10.5	10.4	-1	10.5	10.9	+4
10.5	10.7	+2	10.5	10.6	+1
10.5	10.7	+2	10.5	10.2	-3
10.5	11.1	+6	10.5	10.4	-1
10.5	10.5	0			

TABLE 2.
Recovery of calcium added to human milk.

Calcium found in human milk, mgrms. %	Calcium added mgrms. %	Theoretical value mgrms. %	Found mgrms. %	Calcium found in human milk, mgrms. %	Calcium added mgrms. %	Theoretical value mgrms. %	Found mgrms. %
25.2	5.2	30.4	28.1	25.8	7.0	32.8	33.8
23.6	5.2	28.8	28.5	24.2	7.0	31.2	29.1
21.2	5.2	26.4	26.3				

Conclusion.

Procedures necessary for the application of Yoshimatsu's blood calcium method to human milk have been described. The amount of human milk used for one sample is 0.5 c.c. in the present method.