CHAPTER II

MATERIALS AND METHOD OF PREPARATION

Study on Serum Folic Acid Level.

1. Materials for maintenance and assay of folic acid.

1.1 Stock Culture of Lactobacillus casei.

Lactobacillus casei, ATCC (American Type Culture Collection) No. 7469, obtained from the Food and Nutritional Research Centre, Philippines.

1.2 Microbiological Assay Inoculum Broth Dehydrated.

For cultivation of Lactobacilli for use in microbiological assay. (Fisher Scientific, Fair Lawn, N.J., U.S.A.)

1.3 Folic Acid Assay PGA Broth.

Microbiological Culture Media, (Baltimore Biological Laboratory (BBL), Cockeysville, Maryland 21030, U.S.A.)

1.4 Chemicals

1.4.1 Folic acid (Koch-Light Laboratories LTD., Colnbrook Bucks, England)

1.4.2 Tween 80

1.4.3 Sodium hydroxide (NaOH)

1.4.4 Disodium hydrogen phosphate (Na_2HPO_4)

1.4.5 Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)

1.4.6 Ascorbic acid

1.4.7 Potassium dichromate

1.4.8 Concentrated sulfuric acid

1.4.9 Ethanol

1.4.10 Lysol

1.5 Glassware

Test tubes (2X10 cm.) with plastic caps

Rimless tubes

Volumetric flask 1 liter, 500 ml., 100 ml.

Pipette 0.1 ml., 0.2 ml., 1 ml., 5 ml., 10 ml.

Automatic syringe

Measuring cylinder

Beaker 50 ml., 100 ml., 250 ml., 500 ml.

Dark-brown plastic bottles

Dessicator

1.6 Instruments

Refrigerator 4°C

Deep freezer -20°C

Incubator 37°C

Autoclave

Hot air oven

Centrifuge (International Portable Refrigerated Centrifuge, Model PR-2)

pH meter (Beckman)

Nephelometer (Nepho-Colorimeter, Model 9,

Coleman Instruments Corporation).

2. Method of Preparation.

Maintenance of Stock Culture of Lactobacillus casei.

Lactobacillus casei is maintained in Micro Inoculum Broth or Maintenance Media at 4°C.

One ampoule of the lyophilized culture is transferred to a 10 ml. volume of Sterile Micro Inoculum Broth and incubated at 37°C. for 18 hours.

It is check for purity by plating onto a Blood Agar Medium, and meanwhile is stored at 5°C.

If pure, a drop is inoculated into another sterile 10 ml. volume of Micro Inoculum Broth incubated at 37°C for 18 hours, checked for purity and stored at 5°C. This is to be maintained at refrigerated temperature to be used for assays, and sub-culture into another fresh Micro Inoculum Broth within two weeks.

These two-weekly sub-culture are done so as to maintain the Lactobacillus casei and also to avoid possible mutation. Strict aseptic technique were observed in sub-culturing and inoculating, and the old culture is not discarded until the new culture is proved to be a pure one.

Preparation of Micro Inoculum Broth (Maintenance - Medium).

It is available ready-made in dry form (stored in refrigerator) with pH 6.7

The formula (per liter) is as follow :-

Yeast Extract Bacteriological	20.0
Multi-Peptone	5.0
Glucose	10.0
Monopotassium Phosphate	2.0
Mixed Fatty Acid Ester Complex	0.1

Dissolve 37.1 grams of the dehydrated material in a liter of deionized distilled water.

Dispense 10 ml. of media into tubes. Plug with cotton wool and capped and then sterilize in autoclave at 121°C for 15 minutes.

Check for sterility by absence of cloudiness or growth of bacteria by incubation of all tubes at 37°C overnight.

If sterile store at 5°C until required for use.

Preparation of Folic Acid Assay PGA Broth.

This is available readymade in the form of dry mixture. It is stored at 5°C and once the seal is broken it must be stored over anhydrous calcium chloride in a dessicator in a refrigerator.

It is made up as follow :

Suspend 94 grams of the dry material in a liter of Purified Water U.S.P., to make double strength medium.

Add 0.1 ml. of Polysorbate 80. Mix. Heat with frequent agitation, and boil for one minute. Dispense in five ml. amounts in test tubes.

Ascorbic Acid Phosphate Buffer.

This buffer is prepared fresh each time before use. 1.0 gram of ascorbic acid is dissolved in every 100 ml. of phosphate buffer pH 6.1

Stock Solutions :-

1). 0.1 N Sodium Hydroxide Solution

0.4 gm. of NaOH in 100 ml. deionized water.

2). Phosphate Buffer pH 6.1

Solution (a) : 31.2 gm. $NaH_2PO_4.2H_2O$ + deionized water to 1 liter.

Solution (b) : 28.4 gm. Na_2HPO_4 + deionized water to 1 liter.

Add together 212.5 ml. of (a) to 37.5 ml. of (b) and make up to 1 liter with deionized water.

pH is 6.1

Folic Acid Standards

Three standard solutions of folic acid were prepared and stored between usages at -20°C.

Stock 10^{-4} gm./ml.

10 mg. (dry) folic acid accurately weighed is dissolved in 100 ml. of 20 % ethanol in water (v/v)

with 1 ml. of 0.1 N NaOH added. Store in brown plastic bottles at -20°C in aliquots of 8-10 ml. Prepare fresh every 12 months.

Working 10^{-8}, 10^{-9}, 10^{-10} gm./ml. Folic Acid Standards.

To prepare 10^{-6} gm./ml. Folic Acid.

Dilute 1 ml. of the stock 10^{-4} gm./ml. Folic Acid to 100 ml. with deionized water in volumetric flask.

The working folic acid standards are stored in brown plastic bottles in 50 ml. aliquots at -20°C. Prepare fresh every 6 to 8 weeks. All standard solutions, once defrosted should be discarded to avoid using deteriorated standard.

Preparation of Serum Samples.

Blood is obtained from fasting subjects using acid-washed sterile syringes to ensure freedom from contamination with traces of folate and transfer to acid-washed screw top tubes. The blood is allowed to stand for approximately three hours at room temperature. The clots are rimmed with glass rods, the tubes were centrifuged for five minutes at 3,000 r.p.m. and the supernatant serum aspirated with acid-washed pipettes and frozen at -20°C until assay. On the day of assay, the sera are thawed.

Preparation of Inoculum (L. casei - Suspension)

A 10 ml. amount sterile Micro Inoculum Broth is

inoculated with 1 drop of the latest maintenance 18 hour culture (stored in refrigerator) and incubated at 37°C for 18 hours. This is the 18 hour culture. The Lactobacillus growth is mixed well and 0.5 ml. of this (18 hour culture) is inoculated into another 10 ml. amount of sterile Micro Inoculum Broth. Incubate this for 6-8 hours at 37°C. This is the 6-8 hour culture. 0.5 ml. of this 6-8 hour growth is then inoculated into a 10 ml. amount of sterile single strength assay medium. Mix well to give an even suspension of bacteria. One drop of this suspension (in single strength assay medium) is used as the inoculum for each of the assay tubes.

3. Techniques of assaying folic acid.

All glassware used in the assay must be folate-free, i.e., all tubes, beakers, etc., must have been boiled for 30 minutes in strong blood-dissolving detergent and then must have been rinsed with tap water and distilled water several times.

3.1 The evening before the planned assay, inoculate tube with L.casei for 16-18 hour growth as follows : from the last 16-18 hour growth (store in refrigerator at 4 to 6°C) inoculate 1 drop into 10 ml. of fresh maintenance medium. Place in the incubator at approximately 3 p.m. and let it remain overnight at 37°C.

3.2 First thing in the morning prepare six-to-eight hour growth as follows : take 0.5 ml. of the 16-18 hour growth and inoculate that amount into a fresh tube of 10 ml. of maintenance medium. Shake. Store the 16-18 hour-growth in a refrigerator. Incubate the newly inoculated tube for six-to- eight hours, i.e., till approximately 3 p.m. It is from the six-to-eight-hour growth that the assay tubes will be inoculated.

3.3 Thaw the three P.G.A. standards, which should be always kept in amber or brown plastic bottles, by removing from freezer and placing for completely thawed at room temperature. These standard solutions contains $1X10^{-10}$, $1X10^{-9}$, $1X10^{-8}$ gm./ml. of P.G.A.

3.4 Number assay tubes (2X10 cm. plastic cap, glass tubes) for the L. casei dilution tube (LC tube), for standard curve in duplicate (No. 1,2,....) and for unknown serum.

3.5 Make up buffer-ascorbate by adding 1 gm. ascorbic acid just before use, because ascorbate in solution deteriorates rapidly, to each 100 ml. of phosphate buffer (which is stored on a shelf at room temperature).

3.6 With a 1 ml. micropipetting system put 1 ml. of ascorbate buffer into each tube except the dilution (LC) tube.

3.7 Add 5 ml. double-strength assay medium (stored in brown plastic bottles in refrigerator) to each assay tube

with an automatic syringe.

3.8 Add deionized water with an automatic syringe as follows : 5 ml. to dilution (LC) tube ; 4 ml. to blank control tubes ; 3 ml. to tubes contain 1 ml. of standard solution ; 2 ml. to tubes contain 2 ml. of standard solution and 1 ml. to tubes contain 3 ml. of standard solution.

3.9 Add standard solutions, i.e., known amounts of pteroylmonoglutamic acid. Be sure that they are thawed but not warm ; a tiny remaining sliver of ice should have just dissolved when solutions are used.

3.10 Autoclave the tubes for 30 minutes at 120°C and let them cool for approximately half an hour.

3.11 Get sera (unknown) which are running from freezer. Sera take approximately 45 minutes to thaw.

3.12 When the sera have thawed and tubes have cooled, add 0.1 ml. of sera (unknown) to first unknown tube and 0.2 ml. to second unknown tube. For this, use 0.1 and 0.2 ml. pipettes.

3.13 Inoculate between 3 p.m. and 5 p.m. (remember six-to-eight-hour growth requires six to eight hours).

Put 0.5 ml. of the six-to-eight-hour growth into tube marked 'LC'. This must be done with aseptic technique. Mix thoroughly by rotating tube, or stirring with pipette (sterile). Take sterile 1 ml. pipette, flame tip (briefly). Let tip cool. Fill pipette with solution in freshly-stirred

Reference	Tube No.	Double Strength F.A.A. Medium (ml.)	Ascorbic Acid Phosphate Buffer (ml.)	Folic Acid Std. (gm./ml.) 10^{-10}	10^{-9}	10^{-8}	Dei. Water (ml.)		Serum (ml.)
Blank Control	1 11	5	1	Nil.	Nil.	Nil.	4	Mix. Plug with cotton wool. Autoclave at 120° C for 30 minutes Let cool to room temperature.	
Std. 0.1 ng./ml.	2 12	5	1	1	-	-	3		
Std. 0.2 ng./ml.	3 13	5	1	2	-	-	2		
Std. 0.3 ng./ml.	4 14	5	1	3	-	-	1		
Std. 1.0 ng./ml.	5 15	5	1	-	1	-	3		
Std. 2.0 ng./ml.	6 16	5	1	-	2	-	2		
Std. 3.0 ng./ml.	7 17	5	1	-	3	-	1		
Std.10.0 ng./ml.	8 18	5	1	-	-	1	3		
Std.30.0 ng./ml.	9 19	5	1	-	-	3	1		
Unknown Serum	10	5	1	-	-	-	4		0.1
	20	5	1	-	-	-	4		0.2
LC Tube or Single Strength F.A.A. medium	LC	5	-	-	-	-	5		

'LC' tube. Place one drop in each tube including standard curve tube.

3.14 Since L. casei fall into the facultative anaerobe category, all the assay tubes should be capped tightly during incubation overnight.

3.15 The following morning glance at the bottoms of standard curve tubes.. If the tubes have been incubated long enough a definite increase in the amount of growth will be evident in the tubes with each stepwise higher concentration of pteroylmonoglutamic acid ; if this increase is not evident to the naked eye, incubate for two or more hours.

3.16 Read the assay. The nepho-colorimeter must be turned on approximately 20 minutes before using (warm-up necessary). Nepho-colorimeter must be properly adjusted with blank. Take out a nepho-colorimeter tube. Pour contents of the tubes one by one immediately after vigorous shaking tube into nepho-colorimeter tube, read. Pour content back into tubes. As you go, record as indicated in the data book.

3.17 Plot a graph of the standard curve.

3.18 Calculate the unknown in terms of the standard curve.

4. Materials for the study on the folic acid absorption.

Foliamin (IM 15 mg./ml.)

Takeda Chemical Industries, LTD., Osaka, Japan.

Folic Acid-H^3 (G) Potassium Salt.

Code TRA. 34 ; 250 microcuries ; 1,000 - 5,000 millicuries per millimole.

The Radiochemical Centre LTD., Colnbrook Bucks, England.

Folic Acid (non-active)

Koch-Light Laboratories LTD., Colnbrook Bucks, England.

Sodium Hydroxide

Sterile Distilled Water

Liquid scintillation Counter

702 system, Model 186 A, Nuclear Chicago, Illinois, U.S.A.)

Scintillator :

PPO (2,5-Diphenyloxazol)

Dimethyl-POPOP (2,2'-p-Phenylen-bis-(4-methyl-5-phenyloxazol)

Toluene

Absolute Alcohol

5. Method of Preparation Solutions.

Folic Acid Solution

Solution A : Dissolve 10 mg. folic acid powder (non-active) in 0.5 ml. 0.1 N NaOH. Dilute to 10 ml. with

sterile distilled water.

Solution B : Folic acid-H^3 (G) Potassium salt 250 microcuries is supplied freeze-dried in borosilicate glass ampoules. An ampoule may be opened by lightly scratching the serration with the file provided and carefully snapping off the upper end, dissolve with 2.5 ml. of solution A. Dilute to 10 ml. with sterile distilled water.

Therefore stock solution (B) 1 ml. contains 25 microcuries.

To prepare a single dose for patient.

Stock Solution (B) 1 ml. was diluted to 25 ml. with sterile distilled water. Dispense 20 ml. of this diluted solution to the patient. The remaining 5 ml. was kept as a standard.

Preparation of Scintillating Solution.

Stock Solution : 4 gm. of PPO and 0.05 gm. of dimethyl-POPOP were dissolved in 1 liter of toluene.

Scintillating Solution : before use, added 40 ml. of absolute alcohol to 60 ml. stock solution and mixed.

6. Method of the study on folic acid absorption.

6.1 15 mg. of non-radioactive folic acid (foliamin) is given intramuscularly for 3 consecutive days.

6.2 Test carried out 36 - 48 hours after the last injection.

(1) Fast overnight

(2) 20 μCi. H^3-folic acid in 20 ml. water given orally.

(3) 15 mg. foliamin IM at the same time with (2)

(4) The patient should empty his bladder after drinking the solution.

(5) All urine is collected and pooled for 24 hours after the oral dose and determine for the radioactivity in a liquid scintillation counter. Total activity of the H^3-folic acid excreted in the urine is expressed as the percentage of the oral dose.

Method of Counting.

1. Count background for 5 minutes twice and average.

2. Add 0.4 ml. sample and count for 5 minutes, twice and calculate the sample activity in 0.4 ml. sample (X).

3. Add 0.1 ml. standard into (2) and count for 1 minute, twice and calculated the activity in the standard 0.1 ml. (S).

Calculation :

Total dose given $= \frac{S \times 20}{0.1}$ cpm.

Activity in the urine $= \frac{X \times V}{0.4}$ cpm.

where V = total volume of urine

20 = ml. of dose given

% Excretion $= \frac{X \times V \times 100 \times 0.1}{0.4 \times S \times 20}$

$= \frac{X \times V \times 0.5}{0.4 \times S}$