CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemicals:

1.1 Pure standard folic acid (Cold PGA), (Koch-light Laboratories, Ltd., Coln brock-Bicks-England)

1.2 [3',5',9(N)-H^3] Folic acid, Potassium salt (K-^{3}H-PGA), molecular weight 441.4. Item No. J 14293 Code TRK 212; 250 microcuries with a specific activity of 63 curies per millimole was purchased from the Radiochemical Center Ltd., Amersham, England.

1.3 Liquid Scintillator

1.3.1 PPO (2,5-Diphenyloxazol) anhydrous ($C_{15}H_{11}NO$, MW. 221.3), Sigma No. D-4630

1.3.2 Dimethyl-POPOP [2-2'-p-phenylene-bis (4-methyl-5-phenyloxazol)] ($C_{26}H_{20}N_2O_2$, MW. 392.46), Merck No. 7248

1.3.3 Triton X-100 (Octyl Phenoxy Polyethoxyethanol), Sigma No. T-6878

1.3.4 Toluene, analytical grade ($C_6H_5CH_3$, MW. 92.14), J.T. Baker 9460

1.4 Sodium dihydrogen orthophosphate (Sodium acid phosphate, $NaH_2PO_4.2H_2O$, MW. 156.01),

1.5 di-Sodium hydrogen orthophosphate anhydrous, (di-Sodium phosphate anhydrous, Na_2HPO_4, MW. 141.96),

1.6. Norit 'A' Neutral Pharmaceutical grade decolorizing carbon was obtained from Amed and Chemical Co. Irvington, N.J. 07111,

1.7 Polyvinyl pyrolidone (PVP, MW. 25 000)

1.8 20% Trichloroacetic acid (TCA)

2. Glasswares:

2.1 Beaker, size 100, 250 ml

2.2 Bottle, amber, with stopper, size 15, 125, 1000 ml

2.3 Bottle, with glass stopper, size 125, 500, 1000 ml

2.4 Bottle, vial, with plastic stopper size 2 drams

2.5 Bottle, vial, with plastic screw cap for Liquid Scintillation counter, size 30 ml

2.6 Cylinder, measuring, size 100, 1000 ml

2.7 Flask, volumetric, size 10, 100, 200, 1000 ml

2.8 Pipet, serological, size 0.5, 2.0, 10 ml

2.9 Stirring rods, size length 9"

2.10 Test tube rack for tube size 15 ml

2.11 Tube, centrifuge, size 15 ml

3. Instruments:

3.1 Analytical balance (Harvard trip balance, Ohaus scales Corp. Union N.J., U.S.A.)

3.2 Electrical balance (E. Mettler, type H. 16)

3.3 Deep freezer -20^{o}C (Low temperature creast model CA-280 A, Ohnishi Netsugaku Co., Ltd., Japan)

3.4 Refrigerated centrifuge model PR-2, International equipment Co., USA.

3.5 pH meter (Beckman)

3.6 Mixer (Vortex-Genie, Catalog No. 12-812-VI, Scientific Industries SPFLD, Mass.)

3.7 Pipet filler (Regd. design No. 872235, Griffin and George Ltd., Britain.)

3.8 Automatic pipet with disposable plastic tips for size 100, 500, 1000 microliter (Oxford sampler model Q, Oxford Laboratories Inc.)

3.9 Packard Tri-Carb Liquid Scintillation Spectrometer, model 3375 (Packard Instrument Co., Inc., U.S.A.)

Methods of Preparing Solution for Determining FABP in Sera

1. Phosphate buffer:

1.1 Stock solutions

1.1.1 Solution A (0.2 M Sodium-acid phosphate):

31.202 gm of $NaH_2PO_4.2H_2O$ was dissolved in deionized distilled water and adjusted to 1000 ml in a volumetric flask.

1.1.2 Solution B (0.2 M di-Sodium phosphate):

28.392 gm of Na_2HPO_4 was dissolved in deionized distilled water and adjusted to 1000 ml in a volumetric flask.

1.2 Working solution buffer (0.1 M phosphate buffer, pH 7.4):

20 ml of solution A was added to 80 ml of solution B and diluted with deionized distilled water to 200 ml in a volumetric flask. The pH was 7.4.

2. Standard folic acid (Cold PGA)

2.1 Stock standard PGA solution (50 mg/liter):

50 mg of standard PGA was dissolved in deionized distilled water. 1-2 drop of 0.1 NaOH was added and the volume was adjusted to 1000 ml with distilled water.

2.2 Working standard PGA solution (50 ng/ml):

0.1 ml from stock standard PGA solution (50 mg/l) was diluted with deionized distilled water to 100 ml in a volumetric flask and stored in an amber glass bottle (15 ml) at -20°C. The solution must be thawed before using.

3. Tritiated folic acid (^{3}H-PGA)

3.1 Stock ^{3}H-PGA solution (1751.58 ng/10 ml)

^{3}H-PGA (250 microcuries) which has a specific activity of 63 curies/millimole was diluted with deionized distilled water to 10 ml in a volumetric flask.

3.2 Working ^{3}H-PGA solution (5 ng/ml):

2.85 ml of the stock ^{3}H-PGA solution (1751.58 ng/ml) was diluted with deionized distilled water to 100 ml in a volumetric flask and stored in an amber glass

bottle (15 ml) at -20°C. The solution must be thawed before using.

4. 1% PVP coated charcoal (1 gm%)

1.0 gm of Norit 'A' was mixed vigorously in 20 ml of 1% PVP and then centrifuged at 2000 rpm for 15 minutes. The precipitate was diluted with deionized distilled water to 100 ml and stored in an amber glass bottle.

5. 20 gm% Trichloroacetic acid

40 ml of 50 gm% TCA was diluted with deionized distilled water to 100 ml in a volumetric flask and stored in the glass bottle.

6. Liquid Scintillator

100 ml of dimethyl POPOP was dissolved in 200 ml of toluene. 5.5 gm of PPO was dissolved in 100 ml of toluene. These two solutions were mixed with 330 ml of Triton X-100 in a volumetric flask and a volume of 1000 ml was adjusted with toluene and stored in an amber glass bottle.

Methods of Preparation Serum

1. Normal serum: Sera from female and male blood donor were obtained from Thai Red Cross Society, Bangkok.
2. Serum from patients with malaria, pyrexia of unknown origin (PUO), anemia, opisthorchiasis, cirrhosis of liver, hepatitis, jaundice, hepatomegaly, amoebic liver abscess (ALA) and

carcinoma of the liver (CA. liver) were obtained from Hospital for Tropical Disease, Faculty of Tropical Medicine, Vajira Hospital, Siriraj Hospital and Ramathibodi Hospital.

Blood specimens were left in room temperature for 2-3 hours and centrifuged at 2000 rpm for 5 minutes. The supernatant was stored in a vial at -20°C.

Method for Determination of FABP in Serum Samples (Waxman and Schreiber, 1973)

The experiment was carried out in duplicate. The incubation mixture consisted of 0.4 ml serum; 0.1 ml of ^{3}H-PGA (5 ng/ml); 0.8 ml of phosphate buffer (pH 7.4). The mixture was incubated at 25°C. The unbound ^{3}H-PGA (free folate) was removed by coated charcoal suspension. After centrifugation, the supernatant containing the bound ^{3}H-PGA was counted in Packard Tri-Carb Liquid Scintillation Spectrometer. The FABP content was then calculated.

1. The corrected standard (STD) was the amount of radioactivity present when the test amount (0.5 ng) of ^{3}H-PGA was measured in the absence of charcoal.

2. The buffer supernatant control (SC) was the amount of radioactivity present when the test amount (0.5 ng) of ^{3}H-PGA was measured in the presence of charcoal.

Both the STD and the SC tubes consisted of 1.2 ml of phosphate buffer (pH 7.4) after incubation for 15 minutes before adding 0.1 ml of ^{3}H-PGA solution (5 ng/ml).

3. The serum supernatant control (SSC) was run with each serum samples to determine any radioactivity that was not adsorbable to charcoal. Stable PGA (5 ng) was added to the mixture to block serum binding of ^{3}H-PGA. The remaining ^{3}H-PGA activity not adsorbable to charcoal represented impurities of ^{3}H-PGA and was substrated from the ^{3}H count of each serum incubation mixture. It consisted of 0.7 ml of phosphate buffer (pH 7.4), 0.4 ml of unknown serum and 0.1 ml of cold PGA solution (50 ng/ml) after incubation for 15 minutes before adding 0.1 ml of ^{3}H-PGA solution (5 ng/ml).

4. The serum binding control (SBC) was the amount of ^{3}H-PGA bound to serum and the amount of ^{3}H-PGA which was not adsorbed by charcoal. It consisted of 0.8 ml of phosphate buffer (pH 7.4) and 0.4 ml of unknown serum after incubation for 15 minutes before adding 0.1 ml of ^{3}H-PGA solution (5 ng/ml).

After the second incubation of each tube for 45 minutes at 25^{o}C, then 1.0 ml of 1% PVP coated charcoal suspension was added, except for the STD tubes in which 1.0 ml of deionized distilled water was used instead. The tubes were mixed well and centrifuged for 45 minutes at 1500 rpm in a refrigerated centrifuge at 4^{o}C. 1.5 ml of the supernatant in each tube was precipitated with 0.5 ml of 20% TCA solution. These precipitations were centrifuged as in the first time, but for only 30 minutes. After centrifugation, 1.0 ml of the supernatant was added to 10 ml of liquid scintillator in vail and then counted in a Packard Tri-Carb Liquid Scintillation Spectrometer twice for 5 minutes. The

protocol for determination of serum FABP was shown in table 1.

The formula for calculation of the FABP in term of % ^{3}H-PGA bound/0.4 ml of serum was:

$$\text{FABP \%} = \frac{\text{cpm SBC - cpm SSC}}{\text{cpm STD - cpm SC}} \times 100$$

Calculation

FABP in term of pg/ml

^{3}H-PGA 1.0 ml consisted of ^{3}H 5 ng

^{3}H-PGA 0.1 ml consisted of ^{3}H 5 x 0.1 ng

0.5 ng

unknown serum 0.4 ml was bounded with ^{3}H-PGA 0.5 x FABP% ng

$$\frac{0.5 \times 1000 \times \text{FABP}}{100} \text{ pg}$$

5 x FABP pg

unknown serum 1.0 ml was bounded ^{3}H-PGA $\frac{5 \times \text{FABP}}{0.4}$ pg

12.5 x FABP pg

Table 1. The protocol for determination of serum FABP in human sera

Tube NO.	Buffer pH 7.4 (ml)	unknown serum (ml)	Cold PGA (50 ng/ml) (ml)	mixed and incubated for 15 minutes at 25°C	^{3}H PGA (5 ng/ml) (ml)	mixed and incubated for 45 minute at 25°C	Charcoal (ml)	mixed well and centrifuged 1,500 rpm, for 45 min at 4°C	Total volume (ml)	Super-natant (ml)	20% TCA (ml)
STD	1.2	-	-		0.1		(1.0 ml deionized distilled water)		2.3	1.5	0.5
SC	1.2	-	-		0.1		1.0		2.3	1.5	0.5
SSC	0.7	0.4	0.1		0.1		1.0		2.3	1.5	0.5
SBC	0.8	0.4	-		0.1		1.0		2.3	1.5	0.5