CHAPTER II

MATERIALS AND METHODS

I. Materials

Thai male subjects participated in this study were divided into three groups. The first group was the controls group, composed of twenty men from healthy volunteers. They ranged in age from 30 to 60 years, average 42 years. The second group was the other liver diseases, composed of twenty seven patients. They ranged in age from 25 to 67 years, average 44 years. In this group, they were eleven amoebic liver abscess patients, diagnosed by liver aspiration, five hepatic cirrhosis diagnosed by clinical and biochemical background, seven cholangiocarcinoma and four carcinoma of the head of pancreas. These two groups of carcinoma were documented at the time of surgery.

The third group were thirty patients with primary liver cell carcinoma proven histopathologically. They ranged in age from 20 to 69 years, average 47 years.

Blood was collected using aseptic technique and placed in sterile test tubes. The serum was separated, frozen, and then stored at -70°C pending the various assays.

II. Methods

2.1. Polyacrylamide Gel Electrophoresis (PAGE) using the method of Allen and Moore (1966), modified by Pongpaew *et al.*, 1975.

The patterns of human serum proteins which were separated by PAGE, and the gels were quantitatively measured by using microdensitometer.

Principle:- Polyacrylamide gel is polymerized and cross-linked product of the monomer acrylamide and a cross-linked comonomer, usually N,N-methylenebisacrylamide (Bis.). Certain catalyst-redox systems are used for electrophoresis, such as N,N,N,N-tetramethyl-ethylenediamine (TEMED) and ammonium persulfate. In mixing these components together with buffer a three-dimensional net work is formed into a gel by cross-linking polyacrylamide chains growing side by side in the process of polymerization.

2.1.1. Instruments:-

The equipment used for the electrophoresis were manufactured by Ortec® (Ortec, Tennessae, U.S.A.). Model 4,200 Electrophoresis tank and cells, Model 4,100 pulsed constant-power polarization source. The gels were measured with Ortec Model 4,300 integrating microdensitometer.

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2.1.2. Reagents:-

Stock solutions for gel casting

Solution A: 4.5 g. Tris (hydroxymethyl)-aminomethane (Merck) 7.7 ml IN H_2SO_4

0.06 ml N,N,N,N-tetramethylethylenediamine (TEMED) (Eastman)

H_2O, added to 25 ml (pH 9.0).

Solution B: 2.0 ml Solution A.

0.02 ml N,N,N,N-tetramethylethylenediamine

H_2O, added to 10 ml

Solution C: 8.0 g Acrylamide (Merck)

0.2 g N,N-Methylenebisacrylamide (Bis.)(Merck)

H_2O, added to 25 ml

Solution D: 0.053 g Ammonium persulfate (Eastman)

H_2O, added to 50 ml

Solution E: 0.021 g Ammonium persulfate.

H_2O, added to 10 ml

Note:- The stock solutions for casting the gel are sufficient for two chambers. Solutions A, B and C can be kept for about three or four weeks, whereas solutions D and E should be prepared daily for each gel casting.

Tank Buffer: 31.44 g Tris (hydroxymethyl)-aminomethane 4.372 g boric acid (May and Baker) H_2O, added to 4,000 ml (pH 9.0).

Stock Staining Solution

1.7 g Amido Black 10 B (Merck)

10% Acetic acid added to 1,000 ml

Working Staining Solution:

Stock Staining Solution : 10% Acetic acid = 1:10.

One chamber required 800 ml of working staining solution.

Destaining Solution: 10% Acetic acid.

One chamber required 800 ml of destaining solution.

Composition of 8, 6 and 4.5% Gel.

2.1.2.1. 8 per cent:-

10.2 ml solution A*

10.2 ml solution C*

20.4 ml solution D* to fill up to 59 mm in the cells.

2.1.2.2. 6 per cent:-

2.4 ml solution A*

1.8 ml solution C*

0.6 ml H_2O

4.8 ml solution D* to fill up to 68 mm in the cells.

2.1.2.3. 4.5 per cent:-

1.6 ml solution A*

0.9 ml solution C*

0.7 ml H_2O

3.2 ml solution D* to fill up to 78 mm in the cells.

2.1.2.4. Well-forming gel:-

2.0 ml solution B*

2.0 ml solution C*

4.0 ml solution E*

(* Stock Solutions).

2.1.3. Procedure:-

Before casting the gels, the bottoms of the cells were covered with parafilm® and then the cells were placed into a casting stand. The single gel gradients were prepared as mentioned above.

1. After pouring 8% gel solution to fill up to 59 mm in the cells, 6% gel solution was poured into the cells to fill up to 68 mm; then a water-layer was added as quickly as possible before the onset of polymerization.

Polymerization was sufficient when a sharp boundary appeared between the gel and the water. The water was then sucked out.

2. The 4.5% gel solution was then added to fill up to 73 mm in the cells and water-layering was then required in the same manner as in step 1.

3. The well-forming gel solution was placed along with the well-former in the cells and the well-former was then removed after 10-15 minutes. Twelve wells were available in one cell.

4. The wells were rinsed with buffer solution about 5 times and then refilled with the buffer solution up to the top. Five microliters of serum were applied under the buffer in the wells using a Partigen® dispenser (Behringwerke).

5. The cells were then placed in the buffer tank after filling the lower tank with cooled buffer solution up to the specified mark. In the upper tank the cooled buffer solution was filled up to the specified mark as well and two drops of bromphenol blue were mixed in the upper tank for a running indicator.

During electrophoresis, the pulse rate of the power source was increased from 75 pulse/sec. up to 300 pulse/sec. 15 minutes after starting. A voltage of 325 V. was applied to one cell and 400 V. to the other two cells. During the run, the protein moved from the cathode to the anode. The electrophoresis was terminated when the bromphenol blue boundary reached all the way to the bottoms of the cells.

6. The gel was loosened from the sides of the cell using a steady flow of water through a cannula. Then it was gently removed from the cell using a gel remover plate.

7. The gel was stained in 800 ml of 0.017% W/V amido black in 10% V/V acetic acid within 2 hours in a water bath at 65°C.

8. The gel was destained in 10% V/V acetic acid for 2-3 days and the destaining solution was changed daily.

9. The gels were measured with Ortec Model 4,300 integrating microdensitometer.

10. The concentration of proteins were calculated by comparing the area of the peak recorded by microdensitometer with control serum that was run concurrently in every gel (albumin in control serum with albumin in samples and transferrin in control serum with other protein fractions in samples). The bands produced by electrophoresis were identified according to Felgenhauser (1970), Hoffmeister and Schuett (1972) and Hoffmeister (1974) as shown in Fig. 1 and 2.

2.2. Rocket immuno-electrophoresis

The method of electro-immunoassay (rocket immuno-electrophoresis) introduced by Laurell (1972) was used for quantitative measurement of haptoglobin, α_2-macroglobulin, hemopexin, ceruloplasmin, α_2 HS-glycoprotein, transferrin and Gc-globulin.

2.2.1. Instruments:-

The equipment used for rocket immuno-electrophoresis was an electrophoretic device of the same design as according to Laurell's (1972) with a water cooling system (FR model, Haake) surrounding the apparatus.

A 0-300 V DC power supply (Behringwerke, AG, Marburg, Lahn) was used. Glass plates 100 x 100 x 1.5 mm were obtained from Behringwerke AG, Marburg, Lahn.

2.2.2. Reagents:-

Solutions used for rocket immuno-electrophoresis

2.2.2.1. 1.5% W/V agarose solution.

Agarose Hoechst 1.5 g

Barbital buffer to 100 ml

The agarose solution was heated until it was clear. Fifteen ml of the 1.5% W/V agarose solution were used for one plate.

2.2.2.2. Barbital buffer solution, pH 8.6:-

Sodium barbital	41.2 g
Barbital	8.0 g
Distilled water to	10 1

2.2.2.3. Staining Solution:-

Coomasie brilliant blue	6.25 g
Ethanol 95% V/V	4.5 1
Glacial acetic acid	1.0 1
Distilled water	4.5 1

2.2.2.4. Destaining solution:-

Ethanol 95% V/V	4.5 1
Glacial acetic acid	1.0 1
Distilled water	4.5 1

2.2.2.5. Antibodies (Antisera).

Specific antibodies (antisera), rabbit prepared by Behringwerke were used.

2.2.3. Procedure:-

1. Preparation of antiserum agarose plates:-

Agarose, 0.225 g, was added to 15 ml of barbital buffer, heated until boiling and cooled to 50-55°C. The antibody (antiserum) was then added and mixed well. The amount of antiserum added depended on the kind of protein under investigation as shown below.

Proteins	Antiserum/15 ml agarose solution
Haptoglobin	0.2 ml
α_2-macroglobulin	0.2 ml
Hemopexin	0.2 ml
Ceruloplasmin	0.25 ml
α_2 HS-glycoprotein	0.25 ml
Transferrin	0.3 ml
Gc-globulin	0.3 ml

The antiserum containing gel was then poured onto the plate and spread to a uniform thickness. After gelation, 18 wells were formed with 2.5 well-former.

Note:- The agarose plates were thoroughly cleaned before preparing the antiserum agarose plates. The prepared agarose plates could be kept in a humid chamber only overnight before electrophoresing.

2. Diluted serum samples:-

The dilution of the serum with buffer was such that would result in peaks which were 20-50 mm in height. For the proteins under investigation, the dilutions of serum is shown below.

Proteins	Dilution (serum : buffer)
α_2-macroglobulin	1 : 10
Hemopexin	1 : 10
Ceruloplasmin	1 : 10
α_2 HS-glycoprotein	1 : 10
Transferrin	1 : 10
Gc-globulin	1 : 10
Haptoglobin	1 : 30

Note:- The diluted serum was kept at 4°C for no longer than a week, and was taken out of the refrigerator only once before electrophoresing.

3. Electrophoresis:-

Two gels were placed on the electrophoresis apparatus and connected to the buffer by means of wicks. The apparatus was cooled to 4°C using the water cooling system.

Five microliters of the diluted samples were applied into the wells with Partigen® dispenser with the current already on. After application of the samples, electrophoresis was continued with 8 - 10 Volts/cm for three hours.

After electrophoresis, the gels were pressed with filter paper for 15 minutes, then rinsed in 0.1 M sodium chloride solution for 30 minutes, and washed in distilled water for 15 minutes. The gels were covered with filter paper and dried at 37°C over night.

The following day the gels were stained with 0.0625% Coomasie brilliant blue for 45 minutes and then destained in destaining solution until peaks were clearly distinguished. The concentrations of proteins were calculated by using a standard protein curve.

Note:- For every gel, three or four of standard serum samples (Behringwerke, Marburg, Germany) at different dilutions were run simultaneously with the samples as standard controls.

The concentration of haptoglobin was corrected according to phenotypes:

type 1-1 X 0.6

type 2-1 X 1.3

type 2-2 X 1.5

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