CHAPTER 3

MATERIALS AND METHODS

Criteria for Inclusion the Patients in the Study

1. The subjects were adult patients admitted to the medical wards at Ramathibodi Hospital and Chulalongkorn Hospital. There was no restriction on age, sex and weight of the patients.

2. The patients whose signs and symptoms were most compatible to tuberculous meningitis were included in the study. The diagnosis was based on the following :

2.1 Clinical course was consistent with tuberculous meningitis. Headaches, fever, drowsiness and vomiting were common. The patients also developed symptoms of meningeal irritation such as neck stiffness and Kernig's sign. Signs of cranial nerve involvement (usually ocular palsies, less often facial palsies or deafness) might be presented at the time of admission to the hospital. Occasionally, the disease might be presented with a focal neurological deficit, such as hemiparesis, or with signs of raised intracranial pressure, and rarely with symptoms to the spinal cord and nerve roots (21).

2.2 The most important diagnostic procedure was the examination of the cerebrospinal fluid. Lumbar puncture was performed before the administration of antibiotics to determine the characteristics of CSF (21,67) whether :

a) the CSF was clear or slightly cloudy (22,23,24,26).

b) the CSF was under increased pressure (21,22,23,24).

c) there was an increase in the number of cells, usually varying from 25 to 500 cells per cu mm or more but not more than 1,000 cells per cu mm. These might be all mononuclear or mixture of mononuclear and polymorphonuclear leukocyte, with the former predominately (22,24,26,67).

d) the protein content of the CSF was increased up to about 100 to 200 mg % in most cases, but much higher if CSF blockage occurred around the spinal cord (21,22).

e) the sugar content was reduced to level below 40 mg % (21,22, 24,67).

f) the organism was demonstrable by acid-fast bacillus (AFB) stains of the CSF sediment or on culture, but often it was not (22,24).

2.3 Other diagnostic aids included evidence of active tuberculosis elsewhere in the body such as in the lungs, bone, kidney, etc, and history of tuberculosis contact. It has to be emphasized that these might not be found in some patients (21,22,24,26).

3. The patients had no concomitant diseases except tuberculosis outside the nervous system.

4. The patients received four antituberculous drugs given daily at 9.00 AM.

a. oral doses of isoniazid (INH) 300 mg per day or 5 mg/kg/d.

b. oral doses of pyrazinamide 1-1.5 gm per day or 25 mg/kg/d.

c. oral doses of rifampin 600 mg per day or 10 mg/kg/d.

d. intramuscular doses of streptomycin 0.75-1 gm per day or 20 mg/kg/d.

5. The patients did not receive any other antibacterial drugs during the study.

6. The patients were divided into 2 groups.

a. Group I. The patients were given only antituberculous drugs.

b. Group II. Steroids as oral and/or parenteral forms were given with antituberculous drugs.

7. The number of patients in the study were not less than 10 cases.

Studies

I. Studies of Cerebrospinal Fluid and Serum Concentrations of Antituberculous drugs at Various Intervals During Hospitalization

1. Sample Collection

20.0 ml of clotted blood and 10.0 ml of CSF were obtained 3 hours after administration of antituberculous drugs. The blood specimens were allowed to clot at room temperature for about thirty minutes. The sera were then separated by centrifugation for fifteen minutes at 2,000 revolutions per minute (rpm). The CSF and the sera were kept in the freezer under the temperature of-20 ^{o}c until the assays were performed. In all cases, lumbar punctures were performed only when clinically indicated, but in no case was done purely for the purpose of this study. The day 1 of treatment was the first day of the patients admitted to the hospital and treatment with antituberculous drugs was started. The sample collection in all cases was not on the same day of treatment, but was arranged within each seven-day interval. For example, samples were collected from patient 1 and 2 on day 3, 9 and 4,11 of the treatment, respectively.

So, the sample on day 3 from patient 1 and sample on day 4 from patient 2 were arranged in the same interval (days 1-7) and the sample on day 9 from patient 1 and day 11 from patient 2 were arranged in the same interval (days 8-14).

2. Determination of Antituberculous Drugs in CSF and Serum

The experiments were set out as follows.

2.1 Assay of Isoniazid (68)

2.1.1 Reagents. All chemicals used were analytical reagent grade.

a) Isoniazid stock solution, 10 mg per 100 ml.

Ten mg of isoniazid was dissolved and diluted to 100 ml with distilled water in a 100-ml volumetric flask.

b) Isoniazid plasma reference solutions, 1,2,5 and 10 mcg per ml.

Each of four isoniazid stock solution (0.1, 0.2, 0.5 and 1.0 ml) was diluted to 10 ml with drug-free serum in a 10 ml-volumetric flask.

c) Sodium hydroxide, 4 N

Sixteen grams of sodium hydroxide was dissolved and diluted to 100 ml with distilled water in a 100-ml volumetric flask.

d) Ammonium sulfate, amorphous

e) Extraction mixture

Three volumes of purified n-butanol was mixed with 7 volumes of purified chloroform.

f) Hydrochloric acid, 0.1 N

Eight milliliters of concentrated hydrochloric acid was diluted to one liter with distilled water in a 1-liter volumetric flask.

g) trans-Cinnamaldehyde, 0.04 %

One milliliter of choline-free trans-cinnamaldehyde was diluted to 100 ml with absolute ethanol in a 100-ml volumetric flask. This solution could be kept for 3 weeks at 4°c. Prior to isoniazid determination, 1 ml of this solution was diluted to 25.0 ml with absolute ethanol in a 25-ml volumetric flask.

2.1.2 Assay Procedure

a) Two milliliters of each sample was mixed with one drop of 4 N sodium hydroxide and 2.2 gm of ammonium sulfate. The reference solutions were performed in the same manner as the specimens.

b) Twenty milliliters of the extraction mixture was added and then shaked for 30 min on a horizontal shaker at 145 excursions per minute.

c) The organic extract was separated and filtered through Whatman no. 1 filter paper

d) One milliliter of 0.1 N hydrochloric acid was added to 15.0 ml of the filtrate and the mixture was shaked for 15 min on a horizontal shaker at 145 excursions per minute

e) The aqueous supernatant was separated by using a pasteur pipet and 0.5 ml of it was mixed with 0.15 ml of alcoholic trans-cinnamaldehyde reagent.

f) The absorbance of each sample was determined by spectrophotometer, equipped with a microcell unit at 340 nm.

The mixture of 0.5 ml of 0.1 N hydrochloric acid and 0.15 ml of alcoholic trans-cinnamaldehyde were used as the reference solution.

2.1.3 Calculation

Isoniazid concentration of the specimens was determined from the standard curve.

In each experiment, the isoniazid plasma reference solutions were plotted on graphical paper, using the concentration in mcg per ml as the ordinate and the absorbance of the standard as the abcissa. The straight line obtained from the graph by using linear regression was used as the standard curve to calculate the potencies of isoniazid in the specimens. Example of typical standard curve data for isoniazid was the same as rifampin and streptomycin as shown in Appendix 5.

2.2 Assay of Pyrazinamide (69)

2.2.1 Reagents. All reagents used were analytical grade.

a) Pyrazinamide stock solution, 100 mcg per ml

Ten milligrams of pyrazinamide was dissolved and diluted to 100 ml with distilled water in a 100-ml volumetric flask.

b) Pyrazinamide reference solutions, 5,10,25,50 and 100 mcg per ml.

Each of five pyrazinamide stock solutions (0.5,1.0, 2.5, 5.0 and 10.0 ml) was diluted to 10 ml with distilled water in a 10-ml volumetric flask.

c) Trichloroacetic acid, 10 %

Ten grams of trichloroacetic acid was dissolved and diluted to 100 ml with distilled water in a 100-ml volumetric flask.

d) Sodium nitroprusside, 0.2 % freshly prepared

Twenty mg. of sodium nitroprusside was dissolved and diluted to 10 ml with distilled water in a 10-ml volumetric flask.

e) Sodium hydroxide, 2 N

Eight grams of sodium hydroxide was dissolved and diluted to 100 ml with distilled water in a 100-ml volumetric flask.

2.2.2 Assay Procedure

a) 1.8 milliliters of 10 % trichloroacetic acid was added to each of 3.5 ml of samples, pyrazinamide reference solutions, and blank (distilled water). The contents were stirred and centrifuged at 3,000 rpm for 15 min.

b) Three ml of the protein-free supernatant was treated with 0.5 ml of freshly prepared 0.2 % sodium nitroprusside solution and then 0.5 ml of 2 N sodium hydroxide solution.

c) The tubes were left at room temperature for 15 min and the optical densities were recorded at 495 nm in one cm cells by using a Unicam SP 600 Spectrophotometer.

2.2.3 Calculation

The pyrazinamide concentration of the specimen was determined from the standard curve as in 2.1.3.

2.3 Assay of Rifampin

Concentrations of rifampin in CSF amd serum were determined by a modified agar-well diffusion method (70,71,72,73,74).

2.3.1 The Assay Medium

The formula of the antibiotic medium no. 1 (Difco Penassay Seed Agar, Difco, control no. 722955) used as assay medium was as follows.

Peptone	6.0	g
Pancreatic Digest of Casein	4.0	g
Yeast Extract	3.0	g
Beef Extract	1.5	g
Dextrose	1.0	g
Agar	15.0	g
Water, sufficient to produce	1,000	ml

To rehydrate the medium, 30.5 gm was suspended in 1000 ml of distilled water and heated to boiling until the medium was completely dissolved and the mixture became clear. The medium was then sterilised by autoclaving at 15 pounds pressure (121°c) for 15 min. The pH of the medium after sterilization was 6.6 $\pm$ 0.1.

2.3.2 The Assay Micro-organism

The micro-organism used for the test was Sarcina lutea ATCC 9341. The culture was maintained on the antibiotic medium no. 1 slants, kept in refrigerator and transferred once a week.

The culture was freshly inoculated on the antibiotic medium no. 1 slants and incubated at 37°c for 16-18 hours before each experiment. The Sarcina lutea suspension was prepared by washing the culture from surface of the slant with sterile normal saline solution and adjusted to give a light transmission reading of 25 % against normal saline solution as the blank at 580 nm, with a spectrophotometer, when diluted 1 : 10. The undiluted suspension could be used for up to 15 days when stored in a refrigerator.

2.3.3 Preparation of Assay Plates

Sterile glass petri dishes of 90 mm diameter

(100x15 mm) were used. 0.4 ml of the undiluted Sarcina lutea suspension was added to each 100 ml of the melted-assay medium which was cooled to 45-50°c. The flask was swirled to attain a homogeneous suspension. Then, 15 ml of the seeded agar was placed in each of the required number of plates, and allowed to harden on a level surface at room temperature. In each plate, six agar wells were cut from the seeded agar medium to space equally on a radius of 2.8 cm by using a sterile cork borer with 7 mm diameter and by applying slight negative pressure to remove the plug of agar.

2.3.4 Preparation of the Diluting Solution

The mixture of methanol and M/15 phosphate buffer pH 7.38 in ratio 20 : 80 (V/V) used in diluting reference solutions and samples was prepared as follows :

a) M/15 phosphate buffer pH 7.38

Dibasic sodium phosphate	7.6	gm
Monobasic sodium phosphate	2.1	gm
Distilled water to make	1,000	ml

The buffer was sterilized in autoclave after preparation at 121°c for 15 min.

b) 20 volumes of methanol was mixed with 80 volumes of M/15 phosphate buffer pH 7.38 to give the diluting solution.

2.3.5 Preparation of Rifampin Standard solution

The rifampin working standard with the potency of 1,000 mcg per mg was obtained from Lepetit (Thailand) Co. Ltd. It was kept in a tightly closed container and placed in the desiccator over silica gel and stored in the refrigerator.

The rifampin standard solutions were freshly prepared for each experiment to yield the final concentrations of 0.2, 0.25. 0.3, 0.35 and 0.4 mcg per ml by using the following method.

a) The solution (a_1) of 1,000 mcg per ml was prepared by dissolving 0.100 gm of rifampin working standard in sterile diluting solution to make 100 ml in a 100-ml volumetric flask.

b) The above solution (a_1) was furthur diluted with sterile diluting solution to make five dilutions of rifampin ranging from 0.2 to 0.4 mcg per ml as follows :

1.0 ml of solution (a)+ 9.0 ml of sterile diluting solution

= 100 mcg per ml ..... (a_2)

1.0 ml of solution (a_2)+ 9.0 ml of sterile diluting solution

= 10 mcg per ml .....(a_3)

1.0 ml of solution (a_3)+ 9.0 ml of sterile diluting solution

= 1 mcg per ml ..... (a_4)

1.0 ml of solution (a_4)+ 4.0 ml of sterile diluting solution

= 0.2 mcg per ml.

1.0 ml of solution (a_4)+ 3.0 ml of sterile diluting solution

= 0.25 mcg per ml.

3.0 ml of solution (a_4)+ 7.0 ml of sterile diluting solution

= 0.3 mcg per ml.

1.4 ml of solution (a_4)+ 2.6 ml of sterile diluting solution

= 0.35 mcg per ml.

2.0 ml of solution (a_4)+ 3.0 ml of sterile diluting solution

= 0.4 mcg per ml.

2.3.6 Preparation of the Sample

The specimens were diluted with the same diluent as that of the standard dilution to give the appropriate size of zone

diameter and to be within the above standard curve concentrations (0.2-0.4 mcg per ml). At least three different dilutions (1:5, 1:10, 1:20) were prepared for each serum specimen and two different dilutions (undiluted and 1:2) were used for each CSF specimen.

2.3.7 Assay Procedure

a) Preparation of the Standard Plates

In each experiment, one set of standard curve determination plates was also performed. It contained 4 sets of standard dilutions with triplicate plates, leaving one dilution (the median test dilution, 0.3 mcg per ml) as the reference solution in every assay plates to check the variations of the zone diameters.

For deriving the standard curve, alternate wells on each of 3 plates were filled with 50.0 microliter of the median test dilution of the standard and each of the remaining (i.e.9 wells) was filled with 50.0 microliter of one of the other four dilutions of the standard. The procedure was repeated for the other three dilutions of the standard. The volume of the solution added to each well was transferred with the 50 microliter micropipette (Centaur Sciences, Inc.).

The plates were left at room temperature for 1 hour to allow the antibiotic to diffuse into the medium. Then, they were incubated at 37°c for 18-24 hours. The diameters of the inhibition zones produced by various concentrations of the standard were measured by Antibiotic zone reader (Fisher Scientific Co).

b) Standard Curve Determination

The average standard values in each dilution were corrected with the corrected values obtained from the differences

of the average reference zone in each set from the overall reference zone of the experiment. The corrected standard values were plotted on two cycle semilogarithmic paper, using the concentrations in mcg per ml as the ordinate and the diameter in mm of the inhibition zone as the abscissa. The straight line obtained from the graph by using linear regression was used as the standard curve to calculate the potencies of antibiotics in the specimens, as shown in Appendix 5.

c) Preparation of the Sample Plates.

The frozen specimens were thawed at room temperature. Since the test micro-organism Sarcina lutea was sensitive to rifampin and streptomycin but resistant to isoniazid and pyrazinamide, the specimens were treated with the excess amount of semicarbazide (2 drops of 20 mg per ml of semicarbazide hydrochloride solutions) and incubated at 37°c for 1 hour to inhibit the activity of streptomycin. Then, they were prepared as described in 2.3.6. For each dilution of the specimens, alternate wells on each of 3 plates were filled with 50.0 microliter of the median test dilution of the standard and the remaining (i.e.9 wells) were filled with 50.0 µl of one dilution of the specimens. The procedure were repeated for the other dilutions of the specimens. The plates were then treated in the same manner as 2.3.7.a)..

d) Calculations of the CSF and Serum Concentrations of Rifampin.

The average zone diameters of each specimen dilution were corrected with the corrected values obtained from the differences of the average reference zone in each specimen dilution from the overall average reference zone of the standard curve. These corrected zone diameters of each specimen dilution were used to calculate the concentrations from the linear regression line of the standard.

The exact specimen concentrations were obtained by multiplying the above values with their dilution factors.

2.4 Assay of Streptomycin

Concentrations of streptomycin in CSF and serum were determined by a modified agar-well diffusion procedure (70,72,73,74).

2.4.1 Assay Medium

The antibiotic medium no.1 (Difco Penassay Seed Agar, Difco, control no. 722955) was used as assay medium in the test. The formula and preparation method of the medium were described in 2.3.1.

2.4.2 The Assay Micro-organism

The micro-organism used in the test was Enterobacter cloacae of which characteristics are described in Appendix 4. Preparation of the culture was the same as in 2.3.2. The inoculum of Enterobacter cloacae was adjusted to have a transmittance of 25 % against normal saline solution as the blank when the dilution of stock suspension was 1:20.

2.4.3 Preparation of Assay Plates

The assay plates were prepared as described in 2.3.3. 1.5 ml of the undiluted Enterobacter cloacae suspension was added to each 100 ml of the assay medium.

2.4.4 Preparation of the Diluting Solution

The diluent used in the standard and the sample dilutions was phosphate buffer solution pH 8.0 prepared as follows :

Dibasic potassium phosphate	11.73	gm
Monobasic potassium phosphate	0.523	gm
Distilled water to make	1,000	ml

The buffer was sterilized by autoclaving at 121° c for 15 min.

2.4.5 Preparation of the Standard

Standard streptomycin sulfate powder with potency of 767.05 mcg per mg supplied in a tightly closed vial by Dumex Laboratories was placed in a larger bottle containing silica gel, and stored in the refrigerator.

The standard solution (b_1) of concentration 400 mcg per ml was prepared by dissolving 0.0216 gm of standard streptomycin powder and diluting to 50 ml with sterile distilled water in a 50-ml volumetric flask. The solution was freshly prepared and used in the same day. The solution (b_1) was furthur diluted to give a solution of 10,20,30,40 and 50 mcg/ml as the following schedule.

2.0 ml of solution (b_1) + 6.0 ml of buffer pH 8.0

= 100 mcg per ml ..... (b_2)

1.0 ml of solution (b_2) + 9.0 ml of buffer pH 8.0

= 10 mcg per ml

1.0 ml of solution (b_2) + 4.0 ml of buffer pH 8.0

= 20 mcg per ml

3.0 ml of solution (b_2) + 7.0 ml of buffer pH 8.0

= 30 mcg per ml

2.0 ml of solution (b_2) + 3.0 ml of buffer pH 8.0

= 40 mcg per ml

1.0 ml of solution (b_2) + 1.0 ml of buffer pH 8.0

= 50 mcg per ml

2.4.6 Preparation of the Sample

For each serum specimen, undiluted serum and 1:2 dilution (using phosphate buffer pH 8.0 as diluent) were prepared to give appropriate zone diameters within standard concentration range (10-50 mcg per ml) and for each CSF specimen, undiluted CSF was used.

2.4.7 Assay Procedure

a) Preparation of the Standard Plates

The standard plates were prepared as mentioned in 2.3.7 a). The median test dilution was 30 mcg per ml. The diameters of inhibition zones were measured by vernier caliper.

b) Standard Curve Determination

Determination was done in the same manner as 2.3.7 b).

c) Preparation of the Sample Plates

Enterobacter cloacae was sensitive to streptomycin, but resistant to isoniazid, pyrazinamide and streptomycin. After thawing the specimens, assay procedure was performed as described in 2.3.7 c).

d) Calculations of the CSF and Serum Concentrations of Streptomycin.

The method of calculations was the same as in 2.3.7 d).

3. Analysis of Data

3.1 Concentrations of antituberculous drugs in CSF and serum after 3-hour administration of drugs at various intervals were determined in each patient.

3.2 The correlation between CSF and serum concentrations of each antituberculous drug was analysed by using linear regression.

3.3 The mean concentrations of each antituberculous drug in CSF and serum at various intervals were compared by using analysis of variance.

3.4 The ratio of CSF and simultaneous serum concentration of each antituberculous drug was calculated. The mean ratios of them at various intervals were compared by analysis of variance techniques.

3.5 Concentrations of each antituberculous drug in CSF were compared whether higher than the minimum inhibitory concentration (MIC) of each antituberculous drug against Mycobacterium tuberculosis.

II. Comparative Concentrations in CSF and Serum of Antituberculous Drugs between Patients with and without Concomitant Corticosteroid

1. The mean concentrations of each antituberculous drug in CSF and serum at various intervals were calculated in 2 groups of patients. Then, the mean concentrations of each antituberculous drug in CSF and serum were compared at the same interval between group 1 (concomitantly receiving steroid) and group 2 (not receiving steroid) by Mann-Whitney U-test.

2. The ratio of CSF and simultaneous serum concentration of each antituberculous drug in each group was calculated. The mean

ratios at various intervals in each group of patients were determined and then compared at the same interval between 2 groups of patients by Mann-Whitney U test.

III. Clinical Evaluation of Antituberculous Drugs in Tuberculous Meningitis Patients during Hospitalization.

1. Data Collection.

The patient charts were thoroughly studied and recorded during hospitalization as follows :

1.1 History of illness and history of tuberculosis contact.

1.2 Clinical signs and symptoms : e.g., fever, headache, vomiting, meningeal irritation, etc.

1.3 Characteristics of cerebrospinal fluid such as color, pressure, cell count, glucose content, etc.

1.4 Tubercle bacilli found in CSF by AFB stains or on culture.

1.5 Concentrations of antituberculous drugs in CSF and serum.

1.6 Preparations, dosages and routes of administration of steroids

1.7 Course of illness in the hospital

2. Analysis of Data

Before discharge, the patients were divided into 3 groups depending on the data collection and the physician's judgement.

2.1 The patients were completely recovered. No signs and

symptoms and/or neurologic deficits were observed.

2.2 The patients survived, but were not completely recovered. A variety of neurological sequelae (such as hemiplegia, convulsion, ophthamoplegia, etc) were manifested.

2.3 The patients showed no response to treatment and died during hospitalization.