CHAPTER II

MATERIAL AND METHODS

This thesis is divided in two parts, the laboratory study (in vitro) and the clinical study (in vivo).

A IN VITRO STUDIES

Collected 460 clinically isolated strains of gram negative bacteria, the major problems of serious infections, from patients of three hospital centers in Bangkok (Ramathibodi, Chulalongkorn, Rajvithi). These organism were *Acinetobacter spp.*, *Enterobacter spp.*,+ *Escherichia coli.*,+ *Klebsiella spp.*,+ *Citrobacter spp.*,+ *Indole positive proteus*,+ *Proteus mirabilis*,+ *Pseudomonas aeruginosa*, *Salmonella spp.*,+ *Serratia spp.*+ and *Pseudomanas pseudomallii* (Clinically isolated from Ubolrajthani hospital).

(+ are the organisms of Enterobacteriaceae group)

All of these organisms were collected during 1985 and were kept refrigerated on semi-solid nutrient agar (0.4%).

1. Susceptibility test of gram negative bacteria to piperacilin and other antimicrobial agents by disc diffusion method (Barry, 1976a)

a) Preparation of agar plate

Mueller Hinton agar was used as the test agar. After rehydrated the agar medium was sterilized in the autoclave and then

poured into the sterile plate at temperature about 50°C. They were kept to room temperature before use.

b) Preparation of antibiotic sensitivity disc

Antibiotic discs were recieved from the following companies

- Piperacillin; Lederlle Co.Ltd. Lot No. 410559
- Ticarcillin; Beecham Laboratory Reserch Lot No. 16644
- Gentamicin; Meiji Co.Ltd. Lot No. 690829
- Amikacin; Bristal Bayer Co.Ltd. Lot No. 853
- Cefotaxime; Hoechst Co.Ltd. Lot No. 12401
- Cefsulodin ; Ciba Geigy Co.Ltd. Lot No. 004870
- Ceftazidime; Glaxo Co.Ltd. Lot No. 16922

c) Preparation of inoculum and Test plates

The inoculum were prepared by suspending 4-5 freshly isolated colony in MHB and incubated for 2-8 hours at 35-37°C, then adjusted turbidity by standardization with Mc. Farland turbidity (Tube No. 1) to yield 1×10^8 (CFU/ml).

The prepared inoculums were streaked on the MHA plates, then the sensitivity disc of each antibiotic drugs were placed in the proper position (not more than 7 discs in one plate) and incubated at 37°C overnight.

d) Determination of the sensitivity test

After overnight incubation of these plates, the diameter of clear zone were measured and interpreted the zone sizes by using the standards of NCCLS (Table 1)

Table 1 NCCLS (National Committee for clinical Laboratory stanidards)

		Zone diameter interpretive standards, nearest whole mm			
Sensitivity disc	Disc content (µg)	R	IS	MS	S
Piperacillin	100	< 14	15-17		> 18
Ticarcillin	75	< 11	12-14		> 15
Amikacin	30	< 14	15-16		> 17
Cefotaxime	30	< 14		15-22	> 23
Ceftazidime	30	< 14	15-17		> 18
Cefsulodin	30	< 14	15-16		> 17
Gentamicin	10	< 12	13-14		> 15

Notes : R = Resistance

IS = Intermediate susceptible

MS = Moderate susceptible

S = Susceptible

e) Analysis of data

1) Susceptibility of isolated gram negative bacteria to piperacillin was compared to other antimicrobial agents in percentage of susceptibility and resistance

2) Comparative susceptibility of *Pseudomonas aeruginosa* (from three hospital centers to piperacillin and other antibiotic drugs.

3) Susceptibility test of *Pseudomonas aeruginosa* to piperacillin compared with ticarcillin and determination of cross-resistance between these drugs by statistic method (Regression line)

2. Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs) of piperacillin to gram negative bacteria by broth dilution technique (Barry, 1976b)

a) Preparation of broth medium

Mueller Hintou broth (MHB) was used as the medium. The medium was prepared by dissolving 2.1 g in 100 ml distilled water. The rehydrated broth medium was then sterilized in the autoclave for 15 min at 121℃. The final pH of the broth was approximately 7.4. The refrigerated medium should be used as stock solution for at least a week and warmed to room temperature before use.

b) Preparation of antimicrobic dilution

Preparation of standard powder (961.53 µg/ml potency) was prepared to concentration 2000 µg/ml by dissolve 10.4 mg drug powder in 5.0 ml sterile distilled water. This stock solution could be kept frozen at -20℃ for about a week. Only freshly prepared solution was used in this study. The stock solution was diluted furthur by adding MHB to each of piperacillin solution to yield the concentration of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 µg/ml by the standard two-fold dilution technique.

c) Preparation of the inoculum of isolated strains

The inoculum were prepared by the same method of susceptibility test to yield the required inoculum concentration of 1×10^8 CFU/ml. Then this inoculum was diluted 1:2000 by MHB to 2.5×10^5 CFU/ml. The inoculum effect was tested by 1:20 and 1:200 further dilutions with MHB to yield the inoculum sizes of 0.5×10^7 and 0.5×10^6 CFU/ml, respectively.

In the experiment, 0.5 ml of each antibiotic preparation was added to 0.5 ml of each inoculum of isolated strain with the final concentrations of 256, 128, 64, 32 ..... and 0.125 µg/ml

d) Quality control

The control medium (MHB 1 ml) and the control growth inoculum (0.5 ml + MHB 0.5 ml) were used as the control studies.

Both control and tested studies were incubated about 16-18 hours at 35-37℃.

e) Determination of MICs

After the 16-18 hours incubation, the turbidity of these solutions were detected by index of clear broth in which the growth of bacterial strains was inhibited by antibiotics. The lowest concentration of antibiotics that gave the first clear broth solution, was the minimum inhibitory concentration or MIC.

f) Determination of MBCs

The MBCs were determined by agar-plated techniques. Each clear-broth was subcultured to nutrient agar plates to determine the presence of viable organism. After overnight incubation, the subcultures were examined and the number of colony forming units was measured. The MBCs was defined as the lowest concentration that resulted in99.9% of killing micrcorganisms.

g) A quality control

The MICs and MBCs of piperacillin against *S. aureus* ATCC 25923 were determined to correct the method used.

h) Analysis of data

1) The activity of piperacillin against gram negative in cumulative percentage of MICs and MBCs (µg/ml).

2) Comparison of the activity of piperacillin in MICs and MBCs against 10 types of clinically isolated organisms by statistic method (hypothesis of variance ratio).

3) Activity of piperacillin against gram negative bacteria in $MIC_{50,90}$ and $MBC_{50,90}$ (µg/ml).

4) Comparative activity of piperacillin in MIC and MBC (µg/ml) assay against *Pseudomonas aeruginosa* from three hospitals (Ramathibodi, Chulalongkorn, Rajvithi).

5) The MICs and MBCs of piperacillin against *Pseudomonas aeruginosa* and *Pseudomonas pseudomallii* using the inoculums of 10^6 CFU/ml and 10^7 CFU/ml.

B. IN VIVO STUDIES

1. Study for clinical efficacy and bacteriological response of piperacillin

Children (1 month - 13 years) in the department of paedriatics Ramathibodi Hospital, were studied during January, 1985 through October, 1985.

a) Criteria for selection of patients

1) Clinical evidence of blood disease eg. leukemia, agranulocytosis.

2) Respiratory tract infections eg. pneumonia, bronchopneumonia, empyema, pulmonary abscess.

3) Urinary tract infection eq. pyelonephritis with bacteremia, perinephritic abscess.

4) Skin infections eq. burns complicated with septicemia

5) Soft tissue infections eq. infections resulting from trauma, surgery, or other causes.

6) CNS (central nervous system)

7) Septicemia

All of these patients were not penicillin or cephalosporin hypersensitivities.

b) Drug administration and doses

The administration can be either intravenous or intramuscular injections,1 g vial of piperacillin was dissolved in 5 ml of sterile water for injection. Doses for these serious infections were 200-300 mg per kg daily given every 4-6 hours in children and 100 mg per kg daily given every 4-6 hours in infants.

The durations of treatment were 7-21 days.

c) Data collection

Pre-therapy

- The history of patient
  - present illness
  - chief compliants
  - others
- Diagnosis
  - underlying diseases
  - concurrent diseases

- The previous therapy for this infection
- Site of infection
- Causative organism
- Sensitivity patterns of antibiotics

During therapy

- Antibiotic concurrent therapy
- The chief symptom of patient
- Prognosis of disease
- During therapy culture

Post therapy

- Post therapy culture

d) Conclusion

- Clinical response
  - Cure = Complete remission of signs and symptoms
  - Improvment = Significant reduction of signs and symptoms
  - Relapse = Improvment followed by post Rx deterioration
  - Failure = No demonstrable response to Rx
  - Not evaluate

- Bacteriological response
  - Eradication = Abscence of original pathogen post Rx
  - Marked reduction = Decrease in number of organisms to clinically insignificant level therapy.

- Persistence = Presence of origin pathogen(s) post Rx or at time.
- Indeterminate = Situation in which bacteriologic response could not be evaluated, eg. patient's death or administration of conflicting concomitant antibiotics Rx.

- Overall evaluation of antibiotic
  - Effectiveness
    - Improvement
    - Failure
    - Intermediate
  - Patient tolerance
  - Safety

2. Study of adverse effects

These informations were obtained from patient charts after the ward physicians had completed their daily round and the following laboratory tests that performed routinely during piperacillin therapy :

- Haemoglobin
- Total WBC
  - Neutrophils
  - Eosinophils
  - Basophils
  - Lymphocytes and others
- Platelets count
- Albumin
- Globulin
- SGOT (serum glutamic oxaloacetic transaminase)

- SGPT (serum glutamic palmitic transaminase)
- Alkaline phosphatase
- Total bilirubin
- BUN (Blood urea nitrogen)
- Creatinine
- Others (Urinalysis)
- Complete Urinalysis with microscopic examination of sediments.
- Special tests for specific infections were done as appropriate, for example, X-ray of the chest, bones and joints.

The symptom that was noticed other than the hypersensitivity eq. drug fever, skin rash, also the gastro-intestinal disturbance such as nausea vomiting and diarrhoea, local reaction (eq. phlebitis), allergic reaction and hematological symptoms.

3. Pharmacokinetic study of piperacillin after 2 and 4 g intravenous administration in normal subjects and determination of serum drug level in patients with 200-300 µg/kg/day piperacillin, given intravenously.

a) In normal subjects

subjects : 3 men and 4 womem

Doses : 2 and 4 g IV. bolus

(Average 37.96 ± 4.45 mg/kg/dose and 75.94 ± 8.90 mg/kg/dose)

Age : 27 ± 0.5 years

weight : 53.28 ± 2.8 kg

Blood level at 0-6 h. intervals were determined by collecting sera at specific time

: before injection

: at the end of injection (0 h.)

: 30, 60 min, 2, 4, 6 h. after injection

Urine were collected before and during the periods of 0 to 2 h., 2 to 4 h., 4 to 6 h., 6 to 12 h., and 12 to 24 h. after the piperacillin dose. All urine samples were measured and stored at 20°C before analysis.

b) In patient

subjects : 3 paedriatic patients

Age : 1 month - 3 years

weight : 3 to 28.5 kg (mean 13.8 kg)

Dose : 200-300 mg/kg/day

(Average 45.23 mg/kg/day)

Serum drug level were determined by collecting the serum at 10 min, $\frac{1}{2}$ - 1 h., 2-4 h. and at the end of 6 h. of injection

All of blood samples (in normal subjects and in patients) were collected in microtubes (about 2 ml of blood) and allowed to clot at room temperature for about thirty minutes, then the sera were seperated by centrifugation and frozen at -20°c until the assay was performed within 7 days.

c) Piperacillin assay

Serum piperacillin and urinary excretion concentration were determined by a modified agar well diffusion method with

Sarcina lutea ATCC 9341 as the test organism (Erdberg and Chu, 1973).

1) Preparation of the test organism

S. lutea ATCC 9341 was incubated and allowed to grow on nutrient agar slant at 32-35°C overnight. The organism was then washed out with phosphate buffer in saline (PBS) pH 6 ± 0.05, using sterile glass beads, and adjusted to the turbidity of 32-34% transmittance at 580 nm on Coleman Jr. The bacterial suspension was then kept in the refrigerator until use.

2) Preparation of pour plates

The assay medium was prepared by mixing 30.5 g of the antibiotic medium I with 1000 ml of distilled water. The medium was sterilized by autoclaving at 121°C for 15 min and kept to 50°C for at least half an hour before use. The standardized *S. lutea* suspension 0.5 ml was added to each 100 ml of the above media. Pour plates were made by using 12 ml seeded medium for a 9 mm standard pyrex-petri-dish on critically-leveled surface and allowed to solidify at room temperature and stored in the refrigerator for no longer than 24 hours. The pH of the medium was approximately 6.0

3) Preparation of standard curves and assay of specimens

Standard piperacillin powder (961.53 µg/mg potency) supplied by Lederlle Co .Ltd. (Thailand) was dissolved in sterile distilled water and diluted further by sterile buffer I pH 6 ± 0.5 to yield the final concentrations of 3.5, 3, 2, 1.5 and 1 µg/ml. In each seeded agar plate were created six 4 mm diameter wells. Twenty five µl of each concentration of standard piperacillin and each dilution of serum and urine specimens were filled into the wells of those pour plates in the alternative patterns with the reference

standard concentration of 2 µg/ml. Zone diameter of growth inhibition was measured by vernier calipers after overnight incubation at 32°C. Mean of tripicate reading was used and piperacillin concentration in serum and urine was calculated using standard curve plotted between the zone-diameters and standard piperacillin concentration on semi-logarithmic paper. In case that the deviation was too great to be accepted the assay should be repeated as soon as possible.

The correlation coefficients of the regressions lines varied from $\gamma = 0.9968$ to $\gamma = 0.9973$ in serum and from $\gamma = 0.9788$ to $\gamma = 0.9967$ in urine.

4) Piperacillin assay in the presence of aminoglycoside

If aminoglycosides was the concomitant antibiotic, serum piperacillin concentrations were determined by the use of sodium polyanethol sulfonate (SPS). It was found that SPS could selectively inhibit aminoglycosides and polymyxin antibiotics without any significant inhibitory effect on other commonly utilized classes of antibiotics including penicillins (Edberg *et al.*, 1976). SPS is available as 5% sterile liquid (Grobax; Hoffmann-La Roche Inc., USA) in 10 ml vials or in powders form 1.6 ml of sterile 5% SPS solution was added to each 12 ml of 50 melted antibiotic medium no. I agar. Then 0.5 ml of 32-34% T. (transmission) *S. lutea* suspension was added to each 100 ml of the above medium. Pour plates were made and allowed to solidify. Each plate contained 12 ml of seeded medium. The micro-bioassay was carried out in the same way as previously described. As a control, each plate included a central special well containing the high concentration of aminoglycoside to reassure that the aminogly-coside was entirely inhibited. The slope of the standard curve

obtained from this method was equal to the one got from piperacillin assay without SPS being used.

5) Determination of pharmacokinetic parameters in normal subjects

Serum concentration-time curve of piperacillin was performed by least mean square method of analysis.

In normal subjects, piperacillin serum level data after IV administrations were analyzed by the two compartment open pharmacokinetic model designed for rapid injeciton of a drug into vascular compartment (Wagner *et al.*, 1967).

By using the least squares regression analysis and the method of residuals to the sum of the two exponentials : $C_p^t = Ae^{-\alpha t} + Be^{-\beta t}$ where C_p^t represents the serum concentration at time t after the dose. (Gibaldi and Perier, 1975; Wagner, 1975)

α, β = the first order rate constants of the fast and slow disposition processes, respectively

A,B = the zero-time intercepts of the two component of the the biexponential curves

$$\alpha\beta = k_{21}k_{el}$$

$$\alpha + \beta = k_{12} + k_{21} + k_{el}$$

$$k_{21} = \frac{A\beta + B\alpha}{A + B}$$

k_{12} = the specific rate constant for distribution into peripheral compartment

k_{21} = the specific rate constant for distribution out of the peripheral compartment

k_{el} = elimination rate constant from the body

$t_{\frac{1}{2}}\alpha = 0.693/\alpha$

$t_{\frac{1}{2}}\beta = 0.693/\beta$

$t_{\frac{1}{2}}\alpha$, $t_{\frac{1}{2}}\beta$ = average half life for distribution

Distribution volume were determined as

V_1, V_2 = distribution volume of central and peripheral compartment respectively

Vd_{ss} and $Vd_{(area)}$ = distribution volume at steady state and by area under the curve

$Vd_{(ext)}$ = distribution volume by extrapolation

$AUC_{0-\alpha}$ = area under the serum concentration time 0-α

$AUC_{0-\alpha} = A/\alpha + B/\beta$

$$V_1 = \frac{D}{A + B}$$

$$Vd_{ss} = \frac{k_{12} + k_{21}}{k_{21}} V_1$$

$$V_2 = Vd_{ss} - V_1$$

$$Vd_{(area)} = \beta \frac{D}{AUC_{0-\alpha}}$$

$$= Vd_{ss} + \left(\frac{k_{el} - \beta}{k_{21}}\right) V_1$$

$$Vd_{(ext)} = \frac{D}{B}$$

$$= \frac{V_1(\alpha - \beta)}{(k_{21} - \beta)}$$

Total clearance (Cl_{Tol}) were estimated from

$$Cl_{Tot} = FD/AUC_{0-\alpha}$$

Renal clearance (Cl_R) were calculated from

$$Cl_R = Xu_{0-t}/AUC_{0-t}$$

Non renal clearance (Cl_{NR}) can be derived from

$$Cl_{NR} = Cl_{Tot} - Cl_R$$

Results were standardized to 1.73 m^2 body surface area and presented as mean ± standard error.