CHAPTER III

MATERIALS AND METHODS

Materials

A. Reagent

1. Standard cefoperazone sodium (supplied by Pharma Nueva Co.,Ltd, thailand) Lot No. R8066202. (%purity = 92.99 %)
2. Standard sulbactam sodium (supplied by Pharma Nueva Co., Ltd, thailand) Lot No. 05121411634. (%purity = 88.74$\pm$1.88%)
3. Standard enalapril maleate (supplied by Pharma Nueva Co., Ltd, thailand) Lot No. 06022212792. (%purity = 99.60$\pm$1.11%)
4. Standard rosiglitazone (supplied by Pharma Nueva Co., Ltd, thailand) Lot No. A.041903 (%purity = 100.5%)
5. Methanol HPLC grade (Fisher Scientific, UK) Lot No. 0605570
6. Acetonitrile HPLC grade (Merck, Germany) Lot No. I303991627
7. Diethyl ether AR (Labscan, Ireland) Lot No. 06060200
8. Tetrabutylammonium hydroxide (40% in water) (Fuka, Switzerland) Lot No. 119735, 10706098
9. E-test (AB Biodisk, Soluna, Sweden): cefoperazone/sulbactam(2:1) Lot. No. BG2744, BH1044, BG 1426.
10. Mueller Hinton-2 agar (BioMerieux, France) Lot No. 809676101
11. CTA-medium (Becton Dickinson, France) Lot No. 5196273
12. Tryptic soy broth (Hardy diagnostics, CA, USA) Lot No. 07004

B. Apparatus

1. High performance liquid chromatography (Series 1100, Hewlett Packard, USA)
2. Analytical balance (ME 215s,Sartorious, Germany)
3. Digital pH meter (pH-level2, Inolab, Germany)
4. Sonicator (Elma, Germany)
5. Multi-tube vortex mixer (Smi 2601, USA)
6. Vortex-evaporator (HBI, A Haake Buchler, Germany)
7. Centrifuge (Br4i, Jouan, France)
8. Micro centrifuge (Avanti 30, Beckman, USA)
9. Incubator (Memmert ,Schwabach,Germany) serial no. 840-121
10. Freezer -40^{o}C (ultralow, Sanyo, Japan)
11. Vortex (Scientific industries,Inc, NY, USA) serial no 16563

Study A. Determining probability of target attainment of cefoperazone-sulbactam in hospital-acquired pneumonia patients: Using Monte Carlo simulation

This was an open-label prospective pharmacokinetics study. The subjects were recruited from hospital-acquired pneumonia patients who admitted to the internal medical department, Maharaj Nakorn Chiang Mai hospital. The protocol was approved by the Research Ethics Committee, Faculty of Medicine, and Chiang Mai University.

1. Subjects

Inclusion criteria

1. Patient who was diagnosed with hospital-acquired pneumonia based on American Thoracic Society criteria.
2. The age over 15 years old.
3. Received cefoperazone-sulbactam alone or combination therapy for treatment hospital-acquired pneumonia at recommended dose at least three doses.

Exclusion criteria

1. Pregnant, possibly pregnant, or breastfeeding.
2. History of hypersensitivity reaction to any β-lactam antibiotics and β-lactamase inhibitor.
3. Received cefoperazone-sulbactam within 1 week before enrollment.
4. Patient with evidence or history of hepatic disease (AST and/or ALT > 3 times upper limit) or history of biliary obstruction.
5. Severe renal insufficiency (CrCL<30 ml/min or renal dialysis)
6. Patient with impaired immunologic or hematologic function.

Before each subject's participation in study, informed consent was obtained form the subjects or their parents after explaining purpose of study, the process and the risk-benefit of this study.

2. Drug administration and sample collection

Blood samples were collected after fifth dose of cefoperazone-sulbactam treatment. Approximately five ml of blood samples were withdrawn from a forearm vein via an intravenous catheter at before the start of cefoperazone-sulbactam injection and 10 minutes, 2 and 4 hours after administration. All blood samples were collected in heparined tube, chilled

at 0°C. The blood samples were centrifuged at 3000 rpm for 15 minutes. Plasma were removed and placed in glass tubes. They were immediately frozen at -40°C until analysis.

3. Determination of plasma cefoperazone and sulbactam

3.1 HPLC assay of cefperazone in plasma

3.1.1 Sample preparation

Cefoperazone was extracted from human plasma by liquid-liquid extraction method. An aliquot (250 µl) of plasma sample was transferred to a glass test tube, 25 µl of internal standard (25 µl of 1500 µl/ml rosigitazone in acetonitrile) was added. The mixture was shaken on a vertex-mixer for 1 minute. Add 1 ml of methanol into solution, the mixture was shaken on a vertex-mixer for 10 minutes and centrifuged at 10,000 rpm for 15 minutes. An aliquot was transferred into a glass test tube and evaporated under pressure at 60°C until dryness. The sample was reconstituted by 1000 µl of 5 mM tetrabutylammoniumhydroxide, pH 6.4 and shaken for 1minute on a vertex-mixture. 50 µl of aliquot was injected into HPLC

3.1.2 Chromatographic system

Apparatus : HPLC (series 1100, Hewlett Packard , USA)

Column : The analytical column was a OSD Hypersil® C18, 250x4 mm, 5µm (Agilent Teachnologies, USA)

UV detector : 220 nm

Mobile phases : Acetonitrile: methanol: 5mM tetrabutylammoniam hydroxide (13:9:78), pH 6.8

Flow rate : 1.2/ml

Temperature : 25°C

Retention time : Cefoperazone was approximately 8 minutes

Rosiglitazone was approximately 10 minutes (internal standard)

3.1.3 Preparation of standard solutions

Cefoperazone stock standard solutions were prepared. Cefoperazone was accurately weighed 0.0215 g and dissolved in 10 ml of 50% acetonitrile to give a concentration 2000µg/ml (2 mg/ml) cefoperazone. Dilutions of this solution were made with 50% acetonitrile to give working solutions of 10, 100, 250, 500, 1000 µg/ml, respectively

Rosiglitazone (internal standard) solution was prepared by accurately weighing 0.249 g of rosiglitazone and dissolving in 10 ml of acetonitrile to give a concentration 2,500 μg/ml (2.5 mg/ml). The stock solution and working solutions for cefoperazone and rosiglitazone were prepared on the day of analysis.

3.1.4 Preparation of standard calibration curve

An aliquot (25 μl) of working standard solutions of cefoperazone was spiked to blank plasma (225 μl) to produce a set of calibration standards of 1, 10, 25, 50, 100, and 200 μg/ml, respectively. The peak area ratios of cefoperazone to that of internal standard were fitted to a straight line by linear regression analysis. Calibration standard were prepared on the day of analysis.

3.2 HPLC assay for sulbactam in plasma

3.2.1 Sample preparation

Sulbactam was extracted from human plasma by liquid-liquid extraction method. 500 μl of plasma sample was transferred to glass test tube; 50 μl of 500 μg/ml of enalapril meleate (internal standard) in water was added. The mixture was shaken on vertex-mixer for 1 minute. Then add 200 μl of 1 N HCL and shake on vertex-mixer for 1 minute. The mixture was added by 3 ml of diethyl ether and shaken on vertex-mixer for 10 min and centrifuged at 4,000 rpm for 10 min. An upper layer was transferred into a glass test tube and evaporated by nitrogen gas until dryness (approximately 5 minute). The sample was reconstituted by 500 μl of 5 mM tetrabutylammoniumhydroxide, pH 6.5 and shaken for 1minute on a vertex-mixture. 100 μl of aliquot was injected into HPLC

3.2.2 Chromatographic system

Apparatus : HPLC (series 1100, Hewlett Packard , USA)

Column : The analytical column was a OSD Hypersil® C18, 250x4 mm, 5μm (Agilent Teachnologies, USA)

UV detector : 220 nm

Mobile phases : Acetonitrile: 5mM tetrabutylammoniam hydroxide (25:75), pH 6.5

Flow rate : 1.0 ml/min

Temperature : 25°C

Retention time : Sulbactam was approximately 5 minutes

Enalapril maleate was approximately 6 minutes (internal standard)

3.2.3 Preparation of standard solutions

Sulbactam stock standard solutions were prepared. Approximately 0.0225 g of sulbactam was accurately weighed and dissolved in 1 ml of 0.1 N HCl. The solutions were adjusted by sonicated water to 10 ml to give a concentration of 2000 μg/ml (2mg/ml) sulbactam. Dilutions of this solution were made with deionized water to give working solutions of 5, 10, 50, 100, 500, 750, 1000 μg/ml, respectively.

The enalapril (internal standard) solution was prepared by accurately weighing 0.025 g of enalapril and dissolved in deionized water, then adjusted the volume to 50 ml with deionized water to give a concentration 500 μg/ml

3.2.4 Preparation of standard calibration curve

An aliquot (50 μl) of working standard solutions of sulbactam were spiked to blank plasma (450 μl) to produce a set of calibration standard of 0.5, 1, 5, 10, 50, 75 and 100 μg/ml, respectively. The peak ratios of sulbactam to that of internal standard were plotted against the known concentration of sulbactam and the calibration curve were fitted to a straight line by linear regression analysis. Calibration standards were prepared on the day of analysis.

3.3 Method Validation

The method developments were validated following the Guidance for Industry: Bioanalytical Method Validation of Center for Drug Evaluation and Research (CDER) and Center for Veterinary Medicine (CVM), US Department of Health and Human Services, Food and Drug Administration, 2001.

3.3.1 Selectivity

Cefoperazone: Control blank human plasma from six different sources were analyzed using the same procedure of cefoperazone as described earlier. Each blank sample was tested for potential interfering peaks to ensure that there is no interference to the peaks of cefoperazone and internal standard (rosiglitazone).

Sulbactam : Control blank human plasma from six different sources were analyzed using the same procedure of sulbactam as described earlier. Each blank sample was tested for potential interfering peaks to ensure that there is no interference to the peaks of sulbactam and internal standard (enalapril maleate).

3.3.2 Lower Limit of quantification (LLOQ)

Five determinations of the lowest concentration of standard cefoperazone in plasma and those of sulbactam were analyzed. The LLOQ were tested by examination of the accuracy and precision data. The analyte response at LLOQ should be at least 5 times the response compared to blank plasma response. Analyte peak of these concentrations should be identifiable, discrete, and reproducible with a precision not exceeding 20% and accuracy of 80-120%

3.3.3 Accuracy

The accuracy of an analytical method was determined by replicate analysis of samples containing known amounts of three quality control samples: one within one near the LLOQ (low QC sample), one near the center (medium QC sample), one near the upper boundary of the standard curve (high QC sample). The estimated concentration was the mean of the concentrations obtained from five replicates of three concentrations of quality control samples (QC samples)

For cefoperazone, three concentrations of QC sample were 15,75 and 150 μg/ml for low, medium and high concentration, respectively. For those of sulbactam, the analyte sample concentrations were 1.5,30, and 90 μg/ml. These QC samples were analyzed in five replicates for the drug content. The mean valve should be within ±15% of the actual valve accept at LLOQ, where it should be not deviate by more than 20%

3.3.4 Precision

The precision of an analytical method was measured by assessing the agreement between replicates of three QC sample (low, medium, and high concentration).

3.3.4.1 Within-run precision

For cefoperazone, four concentrations of QC sample were 1, 15,75 and 150 μg/ml for LLOQ, low, medium and high concentration, respectively. For those three concentrations of sulbactam, the analyte sample concentrations were 1.5,30,90 μg/ml. These QC samples were analyzed in five replicates on the same day. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (C.V.) except for the LLOQ, where it should not exceed 20% of the C.V.

3.3.4.2 Between-run precision

For cefoperazone, four concentrations of QC sample were 1, 15,75 and 150 μg/ml for LLOQ, low, medium and high concentration, respectively. For those of sulbactam, the analyte sample concentrations were 1.5,30,90 μg/ml. These QC samples were analyzed in five replicates on three different days. The percent coefficient of variation

(C.V.) of estimated concentration was determined as each concentration level. The precision determined at each concentration level should not exceed 15% of the C.V., except for the LLOQ, where it should not exceed 20% of the C.V.

3.3.5 Recovery

The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the plasma, compared to the detector response obtained for the true concentration o f the pure authentic standard.

For cefoperazone, five determination of three concentration of QC sample (15, 75 and 150 μg/ml) in plasma and in deionized water were analyzed. For sulbactam, five determinations of three concentration of QC sample (1.5,30,90 μg/ml) in plasma and in water were analyzed. Percentage of recovery was calculated by

$$\%\text{Recovery} = \frac{\text{Peak area of analyte extracted from plasma X 100}}{\text{Peak area of analyte unextracted in water}}$$

Recovery of the analyte need not be 100%, but extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible.

3.3.6 Calibration curve

For cefoperazone, a blank sample, zero sample and six concentrations of standard solution of 1, 10, 25, 50, 100 and 200 μg/ml in plasma were analyzed. For those of sulbactam, a blank sample, zero sample and seven concentrations of standard solution (0.5, 1, 5, 10, 50, 75 and 100 μg/ml) in plasma were analyzed. The peak ratios of cefoperazone and sulbactam to those of internal standard were plotted against the corresponding concentration of the analyte. The calibration curves were constructed by linear regression analysis. The coefficient of determination (r^2) should be more than 0.99. The 20% deviation of the LLOQ from nominal concentration and 15% deviation of standards other than LLOQ from nominal concentration should be met.

3.3.7 Stability studies

3.3.7.1 Freeze and thaw stability

Analyte stability was determined after three freeze and thaw cycle. For cefoperazone, three concentration of QC sample were 15, 75 and 150 μg/ml. For those sulbactam, the concentrations were 1.5,30,90 μg/ml. Five aliquots at each concentrations were stored at -40°C for 24 hours and thawed at room temperature. When completely thawed, the samples were refrozen for 12 to 24 hours under the same conditions.

The freeze-thaw cycle were repeated two more times, and then analyzed on the third cycle. The concentrations of freeze thaw sample compared with those of freshly prepared sample. The % deviation of the mean estimated concentration from the zero time should be within ± 10%.

3.3.7.2 Short-term stability

Five aliquots of each the low, medium, high concentration were stored at -40°C for 24 hours and thawed at room temperature for 5 hours. The samples were extracted and analyzed. For cefoperazone, three concentration of QC sample were 15, 75 and 150 μg/ml. For those sulbactam, the concentrations were 1.5,30, and 90 μg/ml. The % deviation of the mean estimated concentration from the zero times should be within ±10%

3.3.7.3 Long-term stability

Long-term stability was determined by storing five aliquots of each of the low, medium and high concentration at -40°C and they were analyzed over a period of 2 and 5 weeks. For cefoperazone, three concentrations of QC samples were15, 75 and 150 μg/ml. For sulbactam, three concentrations were 1.5,30, and 90 μg/ml. The % deviation of the mean estimated concentration from the zero times should be within ± 10%

3.3.7.4 Post-preparation stability

Five aliquots of each the low, medium and high concentration in the processed sample extracts were analyzed after prepared, and kept in the autosampler at 24 hours. For cefoperazone, three concentrations of QC samples were 15, 75 and 150 μg/ml. For sulbactam, three concentrations were 1.5,30, and 90 μg/ml. The % deviation of the mean estimated concentration from the zero times should be within ±10%.

4. Pharmacokinetic analysis

The plasma drug concentrations time data of each dosage regimens were analyzed by non-compartment pharmacokinetic model using the Winnonlin 3.2 program (Pharsight Corporation, USA). Log mean concentration-time profiles were graphed for each subject. The maximum concentration (Cmax) was obtained directly from a plot of concentration-time data. The terminal elimination rate constant (Ke) was obtained by least squares regression anlysis of the terminal phase of the log-linear plot of concentration-time data. Individual half-life values were calculated as 0.693/ke. The Area under the concentration-time curve (AUC) was calculated using the linear-trapezoidal rule. Systemic clearance was estimated as dose/AUC.

5. Determination of MICs distribution

5.1 Microorganisms

Over a 6 month period (July-September, 2006 and May-July, 2007), all true sputum and tracheal secretion isolates of pathogenic *P.aeruginosa* and *A.baumannii* were collected from patients who were admitted at Maharaj Nakorn Chiang Mai hospital. The strains of *P.aeruginosa* and *A.baumannii* were cultered in CTA agar until the test was performed

5.2 Antimicrobial susceptibility

Antimicrobial susceptibility was evaluated by disk diffusion method and the minimum inhibitory concentration (MIC) of cefoperazone-sulbactam was determined by the E-test method according to manufacturer's recommendation and the guidelines of the Clinical and Laboratory Standards (CLSI).

5.2.1 Inoculums preparation

The strains of *P.aeruginosa* and *A.baumannii* were subcultered onto sheep blood agar and incubated for 24 hours at 35°C. Colonies from the second blood agar plate were suspended in broth to a density of 0.5 McFarland.

5.2.2 Disk diffusion method

Disk diffusion tests were performed as described in NCCLS standards M2-L5. The standard inoculums were inoculated on Muller Hinton agar plate (diameter, 100 mm.). Inoculated plates were allowed to dry before the susceptibility discs (cefoperazone-sulbactam 75/30, Oxiod, Oxiod limited, UK) were applied. Plates were incubated at 35°C, and diameters of inhibition zone were measured after 24 hours of incubation.

5.2.3 E-test method

E-test method was performed on Muller-Hinton plate (diameter, 100 mm.). The plates were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. Inoculated plates were allowed to dry before the E-test strips (cefoperazone-sulbactam 2:1, AB Biodisk, Solona, Sweden) were applied to the media. After application of E-test strips (at the same plate of disk diffusion test), plates were incubated at 35°C. MICs were read after 24 hours on the basis of intersection of the elliptical zone of growth inhibition with the MIC scale on the E-test strip.

5.3 Quality control

P.aeruginosa ATCC 27853 and *E.coli* ATCC 25922 were included in the study as control strains.

5.4 Interpretation of susceptibility results

Organisms in the categories of susceptible, intermediate, and resistant were determined by using the breakpoints provide in CLSI guidelines.

Disk diffusion method: For cefoperazone-sulbactam, the breakpoint of ≤15 mm (susceptible), 16-20 mm (intermediate), ≥21 mm (resistant) that are recommended for *P.aeruginosa* and *A.baumannii.*

E-test method: For cefoperazone-sulbactam, the breakpoint of < 16 μg/ml (susceptible), 16-48 μg/ml (intermediate), ≥ 64 μg/ml (resistant) that are recommended for *P.aeruginosa* and *A.baumannii.*

6. Pharmacokinetic-pharmacodynamic analysis ; Monte Carlo Simulation

Pharmacodynamic analyses were conducted via a 5000 subjects Monte Carlo simulation (Crystal Ball; Decisioneering Inc., Denver, Co) for each dosage regimens to estimate the concentration profile after administration. The percentage time that the free drug concentration remained above the MIC (%free T>MIC) was calculated according to an intravenous bolus model that permitted variation in the volume of distribution, half–life and protein binding.

$$\%\ \text{free T>MIC} = \ln \frac{(\text{Dose} \times \text{fu})}{\text{Vd} \times \text{MIC}} \times \frac{t1/2}{0.693} \times \frac{100}{\text{DI}}$$

Where ln is the natural logarithm, Dose is the intermittent dose in milligrams, Vd is the volume of distribution in liters, MIC is the minimum inhibitory concentration in micrograms per milliters, t1/2 is the half life in hours and DI is the dosing interval in hours.

Variability among Vd was assumed to follow log-Gaussian probability distributions during simulation. MIC distributions were built for each population of bacteria based on the frequencies. Variability among fu was assumed to follow a uniform distribution, where all estimates between the ranges provided have an equal likelihood of occurrence.

From these data, the probability of target attainment (PTA) or cumulative fraction of response (CFR) was calculated for each antibiotic regimen. Bactericidal pharmacodynamic breakpoints for cefoperazone-sulbactam were defined as a *f*T>MIC of at least 50%. A dosage regimen was considered optimum if a CFR of 90% or greater resulted. The values were also

calculated for alternate exposures. The CFRs to obtain a T>MIC of at least 10%,20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% were calculated.

8. Statistical analysis

1. Descriptive statistic such as mean and standard deviation were used to describe demographic data and the variables in the study.

2. A normality test for pharmacokinetic parameters were performed to confirm that each parameter were normal distributed. An analysis of various (ANOVA) or Kruskal-Wallis test was used to compare the estimated PK parameters for each dosage regimens studied. A p-value less than 0.05 were considered statistically significant

Study B : Identify factors associated with clinical outcome of treatment hospital-acquired pneumonia with cefoperazone-sulbactam

This is a prospective analytical study to identify factors associated with clinical outcome of treatment hospital-acquired pneumonia with cefoperazone-sulbactam. The subjects were recruited from hospital-acquired pneumonia patients who admitted to the internal medical department, Maharaj Nakorn Chiang Mai hospital. The protocol was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University.

1. Subjects

Cases of *P.aeruginosa* and *A.baumannii* hospital-acquired pneumonia who met the inclusion and exclusion criteria were enrolled to study.

Inclusion criteria:

1. Age over 15 years old.

2. Hospital-acquired pneumonia is diagnosed based on ATS and IDSA guideline with the first episode of HAP infections.

3. Recent sputum culture being positive for *P.aeruginosa* or *A.baumannii* with either susceptible, intermediate or resistance to cefoperazone-sulbactam

4. Receiving cefoperazone-sulbactam alone or combination therapy for treatment HAP, the regimen will not be changed until treatment day 3 (at the earliest).

Exclusion criteria:

1. Pregnant, possibly pregnant, or breastfeeding.

2. History of hypersensitivity reaction to any β-lactam antibiotics and β-lactamase inhibitor.

3. Received cefoperazone-sulbactam within 1 week before enrollment.

4. Patient with evidence or history of hepatic disease (AST and/or ALT > 3 times

upper limit) or history of biliary obstruction.

5. Severe renal insufficiency (CrCL<30 ml/min or renal dialysis)
6. Patient with impaired immunologic or hematologic function.

2. Patients and data collection

All patients who met the criteria were included into study. Hospital medical records were reviewed for information on

2.1 Patient demographics e.g. age, gender, weight, ward of admit

2.2 Clinical data: serum creatinine, liver function test, underlying disease, co-morbidity, clinicals sign and symptoms.

2.3 Microbiological data: Infection focus, organism, co-infection, antibiotic susceptibility and MIC for cefoperazone-sulbactam.

2.4 Pharmacokinetic/Pharmacodynamic data : Cefoperazone and sulbactam blood levels were analyzed by using HPLC with UV detection. Cefoperazone concentrations and %*f*T>MIC were calculated by equation 6

3. Clinical outcome assessment

All eligible patients were assessed for a clinical and microbiological response. All evaluations were performed on day 1, day 3, day 7 or the end of cefoperazone-sulbactam treatment.

Clinical responses were assessed as followed:

Clinical cure: Suandok clinical points of cure were used to determine clinical cure of treatment. The criteria were all these followings

1. No fever (>37,3^{o}C) more than 24 hours
2. Absence or non-purulent sputum
3. No more vasoactive drug more than 24 hours
4. CPIS score<6
5. Chest X-ray shows no progression/cavities/effusion

Clinical improvement: The resolution of all signs and symptoms, continued stable signs upon discontinuation of antibiotic therapy, and no subsequent need of antibiotics for treatment of relapse with the follow-up period or decreased CPIS score.

Clinical failure: Any of the following conditions: persistence or progression of signs and symptoms of infection, development of new active infection, or death because of infection or unchanged or increased CPIS

Microbiological outcome were assessed as followed;

Microbiological cure: Elimination of the *P.aeruginosa* or *A.baumannii* from the site of original isolation (sputum) during completion of therapy or absence of sputum for culture and evaluation.

Microbiological failure: Persistence of the organism, whether or not it had acquired resistance.

4. Stastistical analysis

Chi-squre was used to identify pharmacodynamic variables (%T>MIC, and %free T>MIC) associated with clinical response and microbiological response. Similarly, clinical factors related to patient demographics, medical history, clinical status and antibiotic therapy were tested for associations with treatment outcome. (α=0.05)