Chapter III

MATERIALS AND METHODS

1. Materials

1.1 Medications

Bacampicillin 400 mg tablet, provided by Astra Läkemedel AB, Södertalje, Sweden. It was available as hydrochloride salt. (Batch no. HH 220)

Ampicillin B.P. 250 mg capsule (Batch no. 4042)

Ampicillin B.P. 500 mg capsule (Batch no. 4393), manufactured by Beecham Pharmaceuticals, England. They were commercially available.

The actual contents per unit dosage of the drug were analysed using microbiological method to show any deviation of intended amount.

1.2 Reagents

a) Standard ampicillin powder (Lot no. 841101; potency 924 mg/g) provided by Astra Läkemedel AB, Södertalje, Sweden.

b) Culture medium

Antibiotic Agar No. 1 (Difco Laboratory)

Ingredients per litre :

Bacto-Beef Extract	1.5 g
Bacto-Yeast Extract	3 g
Bacto-Casitone	4 g
Bacto-Peptone	6 g
Bacto-Dextrose	1 g
Bacto-Agar	15 g

Bacto-Nutrient Agar (Difco Laboratory)

Ingredients per litre :

Bacto-Beef Extract	3 g
Bacto-Peptone	5 g
Bacto-Agar	15 g

c) Diluents

Potassium phosphate, monobasic crystal reagent A.C.S.
(Metheson Coleman & Bell Manufacturing Chemists)

Potassium phosphate, dibasic
(May and Baker Ltd.)

Potassium hydroxide, reagent A.C.S.
(Matheson Coleman & Bell Manufacturing Chemists)

Normal Saline Solution, 0.9%

(Abbott Pharma Ltd.)

Sterile Human Plasma

(Supplied from Blood Bank, Ramathibodi Hospital)

d) Test organism

Sarcina Lutea ATCC 9431

2. Methods

Subjects

Fourteen healthy adult volunteers, seven males and seven females participated in this study. Standard laboratory biochemical screening was performed prior to the study for all subjects to ensure the absence of any significant hepatic or renal disturbance or known diseases of gastrointestinal tract. The subjects were shown to be in good physical condition with normal blood and urine laboratory values which were accessible in appendix A. They also had no histories of hypersensitivity reaction to penicillins or cephalosporins. All subjects were fully informed of the nature of the study, the potential role of ampicillin as an allergen and the possible effect on bacterial flora. They were permitted to take no antibiotic for at least one week preceding the study, and no drug otherthan the required doses of ampicillin or bacampicillin was taken during the study.

Drug Administration

The drugs were given orally in a single dose

Bacampicillin 400 mg

Bacampicillin 800 mg (Two tablets of bacampicillin 400 mg)

Ampicillin 250 mg

Ampicillin 500 mg

The doses were taken under supervision with 200 ml water in the fasting condition or with the test meal.

Experimental Design

The study was conducted in a randomized crossover design at four different occasions. Each subject received the drug in a randomized order, implying that different treatments were given to the subjects on the same day, and with a one-week 'washout' period between administrations. The subjects were fasted from at least mid-night before each treatment and were permitted to have no food or fluid intake, apart from the test meal until two hours after drug administration. At the beginning of the treatment, the subjects were instructed to empty their bladders and then received one of the following treatments.

Treatment A_1 : Bacampicillin 400 mg, fasted

The subjects received one tablet of 400 mg bacampicillin orally with 200 ml water.

Treatment A_2 : Bacampicillin 400 mg, non-fasted

The subjects were given the test meal and immediately took one tablet of 400 mg bacampicillin with 200 ml water.

Treatment B : Bacampicillin 800 mg, fasted

The subjects received two tablets of 400 mg bacampicillin orally with 200 ml water.

Treatment C : Ampicillin 250 mg, fasted

The subjects received one capsule of 250 mg ampicillin orally with 200 ml water.

Treatment D : Ampicillin 500 mg, fasted

The subjects received one capsule of 500 mg ampicillin orally with 200 ml water.

All subjects received treatment A_1, C, D. Seven subjects were randomly designed to received treatment A_2 and the remainder seven subjects received treatment B. Thus, each subject received four different treatments as shown in Table 1.

Table 1 : Treatment schedule

Subject no.	Week 1	Week 2	Week 3	Week 4
01	C	D	A_1	B
02	D	A_1	A_2	C
03	A_1	B	C	D
04	A_1	B	C	D
05	D	A_1	A_2	C
06	C	D	A_1	A_2
07	C	D	A_1	B
08	B	C	D	A_1
09	D	A_1	A_2	C
10	D	A_1	A_2	C
11	A_1	B	C	D
12	B	C	D	A_1
13	D	A_1	A_2	C
14	A_2	C	D	A_1

Two hours after administration, a standardized light breakfast was given to subjects receiving any treatment except treatment A_2. The subjects were instructed to take enough water during the study in order to produce sufficient urine samples at the sampling time.

Sample Collection

Blood samples (4-5 ml) were drawn from the antecubital vein into additive-free tube before drug administration and at

0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0 hours after administration of ampicillin and at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 6.0, 8.0 hours after administration of bacampicillin respectively. Blood samples were allowed to clot at 4°C for 1-2 hours. After centrifugation, the sera were collected and kept at -60°C until analysed.

Complete urine collection was done. The subjects were instructed to urinate immediately before drug intake and all urine passed thereafter was collected at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 hours. The volume of each urine sample was measured and recorded. An aliquot portion (5 ml) was immediately kept frozen at -60°C until analysed. All samples were analysed within one week.

Determination of Ampicillin Concentrations in Serum and Urine Samples

The concentrations of ampicillin in serum and urine samples were determined microbiologically by agar-well diffusion method using Sarcina lutea ATCC 9341 as the assay organisms.

1. Preparation of the Standard

For serum specimens, the standard solutions of ampicillin were prepared using Sterile Human Plasma (SHP) as diluent, but for urine specimens the Sörensen's Phosphate buffer pH 7.0 was used. The samples were diluted to appropriated concentrations with the same diluent used in preparing the standard solutions.

1.1 Preparation of diluting solution

The Sorensen's Phosphate buffer pH 7.0 was prepared by dissolving potassium phosphate, dibasic 24.3 g and potassium phosphate, monobasic 56.0 g in distilled water in a one-litre volumetric flask. After dissolved, diluted with distilled water to volume and mixed. The solution was autoclaved at 121°C for 15 minutes. This stock solution was 0.5 M Phosphate buffer pH 7.0, which was diluted ten times with sterile distilled water before used.

1.2 Dilution of the standard solution

The stock standard solution was prepared by dissolving 10.82 mg of an accurately weighed standard ampicillin powder, equivalent to 10 mg ampicillin in 10 ml sterile distilled water. After the powder was completely dissolved, the stock standard solution having a concentration of 1000 mcg/ml was obtained. From the stock solution, a series of dilution of standard solution for urine specimens with concentration range 0.03 - 0.20 mcg/ml was prepared by the following procedure :

10.82 mg of standard ampicillin powder + 10 ml sterile distilled water = 1000 mcg/ml (a)

0.2 ml of (a) + 9.8 ml of buffer pH 7.0 = 20 mcg/ml (b)

1.0 ml of (b) + 9.0 ml of buffer pH 7.0 = 2 mcg/ml (c)

0.5 ml of (c) + 4.5 ml of buffer pH 7.0 = 0.20 mcg/ml

0.5 ml of (c) + 9.5 ml of buffer pH 7.0 = 0.10 mcg/ml (d)

4.0 ml of (d) + 1.0 ml of buffer pH 7.0 = 0.08 mcg/ml

1.5 ml of (d) + 1.5 ml of buffer pH 7.0 = 0.05 mcg/ml

1.5 ml of (d) + 3.5 ml of buffer pH 7.0 = 0.03 mcg/ml

For serum specimens, a series of dilutions of standard solution ranging from 0.03 mcg/ml to 0.15 mcg/ml was prepared by the following procedure :

0.2 ml of (a) + 9.8 ml of SHP = 20 mcg/ml (i)

1.0 ml of (i) + 9.0 ml of SHP = 2.0 mcg/ml (ii)

0.6 ml of (ii) + 9.4 ml of SHP = 0.15 mcg/ml

0.5 ml of (ii) + 9.5 ml of SHP = 0.10 mcg/ml (iii)

4.0 ml of (iii) + 1.0 ml of SHP = 0.08 mcg/ml

1.5 ml of (iii) + 1.5 ml of SHP = 0.05 mcg/ml

1.5 ml of (iii) + 3.5 ml of SHP = 0.03 mcg/ml

2. Preparation of test organism

Sarcina lutea ATCC 9431 was inoculated and allowed to grow on nutrient agar slant at 32-35^{o}C overnight. The bacterial suspension was prepared by washing out the organism from the nutrient agar slant using sterile normal saline solution. The stock suspension was diluted with the same diluent and the concentration was adjusted by determining the transmittance at 580 nm with a spectrophotometer. The standardized inoculum should

have a transmittance of 32-34% against saline as the blank.

3. Preparation of assay medium

The assay medium was prepared by suspending 30.5 g of antibiotic medium No. 1 in one litre of distilled water. The mixture was heated to boil with continuous stirring until the medium was completely dissolved. The medium was then sterilised by autoclaving at 121°C for 15 minutes. The pH of the mixture was 6.6 ± 0.1.

4. Preparation of Plates

The assay medium was melted and kept at 45-50°C for at least half an hour before used. Then, 0.5 ml of Sarcina lutea suspension per each 100 ml assay medium was added. To prepare assay plates, sterile petri dishes (size 15 x 100 mm.) were used and 12 ml of seeded medium were placed in each of the required number of plates, and allowed to solidify on a perfectly horizontal surface at room temperature.

5. Preparation of Samples

The ampicillin concentration of all specimens were roughly estimated and diluted if necessary, to given the appropriate size of zone diameter. Sterile Human Plasma was used as diluent for serum specimens, for urine specimens the phosphate buffer pH 7.0 was used. At least two different dilutions were prepared for each specimen and the concentration of each specimen was determined in triplicate.

6. Assay Procedure

The hardened agar on each plate was cut out within a circle at equal distance to make six equal wells (approximately 4 mm. in diameter per each well). Three wells in each plate were filled with one dilution of standard solution or diluted serum or urine specimen, 25 microlitres per each well, while the other alternate three wells were filled with the median concentration of standard solution (0.08 mcg/ml). The plates were placed on the horizontal surface at room temperature for at least half an hour to allow the standard or sample solutions to diffuse into seeded agar. After incubation at 32°C overnight, the diameters of the inhibition zones were measured using a vernier caliper. The correction factor was calculated by subtracting the mean inhibition zone of the mean concentration of standard solution obtained in each plate from that obtained from the total plates.

The ampicillin concentrations in serum and urine samples were determined from the standard curve which was the regression line ($Y = a e^{bx}$) obtained from the plot of the inhibition zones (X in mm.) versus the concentration of the standard solutions (Y in mcg/ml)

Pharmacokinetic Analysis (74-76)

In the present study, it was proposed that the time course of ampicillin in serum and urine for each subject could be well described by a one-compartment open model with first

order rate constants. The goodness of fit was tested by comparing the experimental data with those calculated theoretically. The coefficient of variation (C.V.) was calculated to indicate the difference between these two sets of data. The smaller the coefficient of variation the better is the fit of the kinetic model. An example of the calculation of C.V. was shown in APPENDIX B. The equation used to predict the serum concentrations of a drug which behaves like a one-compartment model with first order input is as follow :

$$C_t = \frac{Ka\,F\,D}{V_d(Ka - Ke)}(e^{-Ket} - e^{-Kat}) \qquad \text{(1)}$$

where, C_t is the serum concentration at time t, F is the fraction of the dose, D, of the drug which is absorbed. V_d is the drug distribution volume in the body. Ka and Ke are the first-order rate constants for absorption and elimination respectively.

A plot of the natural logarithm of serum concentrations versus time yields a biexponential curve, the terminal portion of which is linear with the slope equal to Ke. The serum half-life ($t_{\frac{1}{2}}$) was then calculated from the following equation :

$$t_{\frac{1}{2}} = \frac{\ln 2}{Ke} \qquad \text{(2)}$$

The absorption rate constant (Ka) was determined from the same plot using the method of residual, that is

$$\frac{KaFD}{V_d(Ka - Ke)}e^{-Kat} = \frac{KaFD}{V_d(Ka - Ke)}e^{-Ket} - Ct \qquad \text{(3)}$$

The area under the serum concentration-time curve was calculated using the trapezoidal rule and the rest area from the last sampling time to infinity was estimated from the following equation :

$$AUC^{t_x \longrightarrow \infty} = \frac{Cx}{Ke} \qquad \text{(4)}$$

where, Cx is the serum concentration at the last sampling time, t_x

The apparent volume of distribution (Vd) was calculated using the following equation :

$$Vd = \frac{D.F}{Ke \,.\, AUC} \qquad \text{(5)}$$

The total clearance (Cl_T), which characterized the clearing of the hypothetical plasma volume of drug was calculated from the following equation :

$$Cl_T = Ke \,.\, Vd \qquad \text{(6)}$$

The time required to reach the peak concentration (t_p) and the peak height (C_p) were also calculated using the equations :

$$t_p = \frac{1}{Ka - Ke} \ln \frac{Ka}{Ke} \qquad \text{(7)}$$

$$C_p = Ae^{-Ket_p} - Ae^{-Kat_p} \qquad \text{(8)}$$

It is sometimes feasible to analyse the pharmacokinetic parameters from urinary excretion data especially for the drug

which is excreted mainly unchanged (77)

$$\frac{d\,Xu}{dt} = k_e\, X_o\, e^{-Ket} \qquad \text{(9)}$$

From equation (9), a semilogarithmic plot of the excretion rate of the unmetabolised drug ($\frac{d\,Xu}{dt}$) versus time results in a biexponential curve of which terminal portion is linear. From the slope of this linear portion, the Ke was estimated. The urinary excretion rate constant, k_e was obtained by dividing the antilogarithmic of the intercept by the dose (X_o). The absorption rate constant, Ka could also be determined using the method of residuals. The Ke obtained from this urinary excretion rate plot should have the same value as the Ke obtained from a semilogarithmic plot of the serum concentrations versus time. Thus, the elimination rate constant of the drug could be obtained either from serum concentrations or urinary excretion rate plot.

An alternative way to determine the elimination rate constant from the urinary excretion data could be accomplished by the sigma-minus method.

$$\text{Since,}\quad \ln (X_u^{\infty} - X_u) = \ln X_u^{\infty} - Ket \qquad \text{(10)}$$

A plot of the natural logarithm of the amount of unchanged drug remaining to be excreted, $\ln (X_u^{\infty} - X_u)$ versus time is linear which its slope represents the Ke.