MATERIALS AND METHODS

Chemicals and drugs

Chemicals

Absolute acetronitrile and methanol were purchased from J.T Baker Company (USA). Hexadecyltrimethylammonium bromide (HDTA), an ion-pairing agent was purchased from Sigma Chemical Company (USA). Dipotassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck Company (Germany).

Drugs

Standard powder of ceftriaxone was purchased from Sigma Chemical Company (USA). Intramuscular ROCEPHIN preparations, 250 and 1,000 mg in a vial container (Roche, Switzerland) were purchased from the Maharaj Nakhon Chiang Mai Hospital (batch numbers 1663,1734, 1774 and 1874). The 250 and 1,000 mg vial preparations of CEF-3 (Siam Pharmaceuticals, 171/1 Soi Choke Chai Ruammitr, Vibhavadi-Rangsit Road, Bangkok 10900) for intramuscular injection, were obtained from private clinics and from the Maharaj Nakhon Chiang Mai Hospital (batch numbers COXB 016, COXB 022, COXG 006, COXK 117, COXK 119 and COXL 120).

Subject selection criteria

Inclusion criteria

Twenty healthy Thai volunteers between 18-32 yr of age (median 24.5 yr) were recruited in the study (Table 1 and 2). All subjects showed no evidence of any illness or underlying disease by a physical examination and clinical laboratory tests including complete blood count (CBC), liver function test (LFT), blood urea nitrogen (BUN) and creatinine (Cr). At least one week before and during the study, each subject was instructed to be abstinent from any drugs as well as cigarettes, alcohol and caffeine beverages that might interfere with the study (Kwon et al., 1985; Melet et al., 1989; Melet et al., 1991 and Brown, 1993). Female subjects were at low risk of conception (sterile or using an adequate method of contraception) and were not pregnant at the time of study (confirmed by a urine pregnancy test).

Exclusion criteria

Subjects with a history of severe adverse reactions to cephalosporins and/or penicillins and any evidence of underlying diseases suggested by a physical examination and / or abnormal clinical laboratory tests (LFT, renal function and/or hematological profile), female who might be pregnant (positive urine pregnancy test) or at a high risk of conceive (nonsterile and without adequate method of contraception), were excluded from the study.

Table 1 Demographic data of subjects receiving 250 mg dosage of ceftriaxone .

characteristics	Subject number								
	1	3	6	7	9	11	13	16	18
Age (yr)	24	25	25	26	22	23	30	26	20
Height(cm)	150	162	160	160	180	160	155	152	179
Weight (kg)	46	53	51	49	75	45	50	44	59
Hct (%)M=40-50 F=36-45	13.8	124	13.3	14	15.9	13.2	14	12.1	13.9
Hb(10-15gm/dL)	40	36	39	35	46	37	40	36	43
WBC(5-10 /cu.mm)x1000	7000	7300	9800	7000	5700	3800	8000	6300	6900
AST(GOT)(3-35u/L)	16	20	15	20	18	18	19	14	23
ALT(GPT)(7-33 u/L)	12	17	11	11	12	13	22	28	27
Bilirubin Total (0.2-1mg/dL)	0.65	0.96	0.95	0.8	0.73	0.69	1.61	0.6	0.8
Direct (0-0.2 mg/dL)	0.15	0.26	0.31	0.5	0.21	0.1	0.19	0.1	0.2
Platelets	Adequate								
Creatinine(0.6-1.7mg/dL)	0.5	0.6	0.6	0.6	0.8	0.7	1.1	0.9	1.2
BUN(7-27 mg/dL)	13.0	17.0	10.0	14.0	9.0	10.0	13.0	9.0	11.0

Table 2 Demographic data of subjects receiving 1000 mg dosage of ceftriaxone.

Characteristics	Subject number										
	0	2	4	5	8	10	12	14	15	17	19
Age (yr)	28	26	21	32	24	18	21	30	26	24	21
Height(cm)	154	150	166	177	167	150	170	174	150	173	173
Weight (kg)	48	52	85	75	67	52	60	60	49	75	61
Hct (%)M=40-50 F=36-45	13	12.5	15.6	13.5	16.5	13.3	16	16.9	11.1	14.8	15
Hb(10-15gm/dL)	39	36	44	40	47	38	45	48	35	45	44
WBC(5-10 cu.mmx1000	8900	5800	9400	8100	9000	6900	8000	6800	7900	7800	8100
AST(GOT)(3-35u/L)	16	17	20	21	17	13.0	28	19	11	16	18
ALT(GPT)(7-33 u/L)	13	17	30	39	32	7.0	27	26	25	35	36
Bilirubin Total (0.2mg/dL) Direct (0-0.mg/dL)	1 0.4	0.53 0.15	1.0 0.12	1.25 0.19	1.2 0.25	0.8 0.22	1.2 0.22	1.07 0.25	1.2 0.7	0.6 0.2	1.6 0.2
Platelets	Adequate										
Creatinine(0.6-1.7mg/dL)	0.8	0.6	1.0	1.4	1.2	8.0	0.6	0.9	0.7	1.3	1.3
BUN(7-27 mg/dL)	13	12	16	17	0.25	1.0	6	18	5	6	11

Study design

This study was a double blind, randomized, two-period crossover design. Treatment for each subject was randomized through a computer-generated list. Only an investigator, who did not participate in the subject care and/or the measurement of drug levels, kept the code and prepared the administered drug during visit 2 and visit 3. Other investigators as well as all subjects, were blind. The randomization was shown in Table 3. The study protocol was approved by The Human Experimentation Committee of the Faculty of Medicine, Chaing Mai University.

Study protocol

Visit 1 : Screening visit:

Each subject had a complete medical history and physical examination performed by a physician. Routine blood tests including CBC and differential, LFT, BUN and Cr were performed. All subjects signed a written informed consent. Vital signs were recorded routinely at the beginning of each visit and periodically at intervals during the study visit.

Visit 2 :

Each subject was randomized to receive an intramuscular preparation of either 250 or 1,000 mg of ROCEPHIN or CEF-3 preparation. An intravenous catheter connected to an injection plug was

Table 3. Randomization of treatment

Subject number	Treatment assigned	
	First visit	Second visit
00	B2	A2
01	A1	B1
02	B2	A2
03	B1	A1
04	A2	B2
05	A2	B2
06	B1	A1
07	A1	B1
08	B2	A2
09	B1	A1
10	A2	B2
11	A1	B1
12	B2	A2
13	A1	B1
14	A2	B2
15	B2	A2
16	B1	A1
17	A2	B2
18	A1	B1
19	B2	A2

Note A1 ROCEPHIN 250 mg
A2 ROCEPHIN 1,000 mg
B1 CEF-3 250 mg
B2 CEF-3 1,000 mg

placed at a forearm for blood sample collections. Five mL aliquots of whole blood were collected from the injection plug at 0, 15, 30, 45 min, 1, 2, 3, 4, 6, 8 and 24 hr and allowed to clot at room temperature. Urine samples were collected during 0-2, 2-4, 4-8, 8-12 and 12-24 hr after the drug administration. Blood samples were centrifuged for 5 min at 2,600 rpm to separate the serum. The serum and urine were then kept at -70°C until analysis for ceftriaxone concentrations with use of a high performance liquid chromatography (HPLC).

Visit 3 :

Same procedures as the previous visit (visit 2) were performed except that the different preparation of ceftriaxone (but the same dose) would be administered. The time interval between visit 2 and 3 was at least 1 week.

Quantitation of ceftriaxone concentrations

1. HPLC methods

The system consisted of an DUG-3A degasser and LC-10 AS pump connected to a SPD-10A UV-VIS detector. Seperation was performed at 40°C on alphasil C_{18} reverse-phase column (0.46 by 25 cm) with a precolumn. The mobile phase was pumped through the column at 1 mL/min and monitored at a wavelength of 280 nm. Peak areas of the chromatogram were integrated with use of a C-R 6A chromatopac. The conditions for HPLC were modified from the

methods previously described (Granich and Krogstad, 1987; Jungbluth and Jusko, 1989).

1.1 Selection of mobile phases

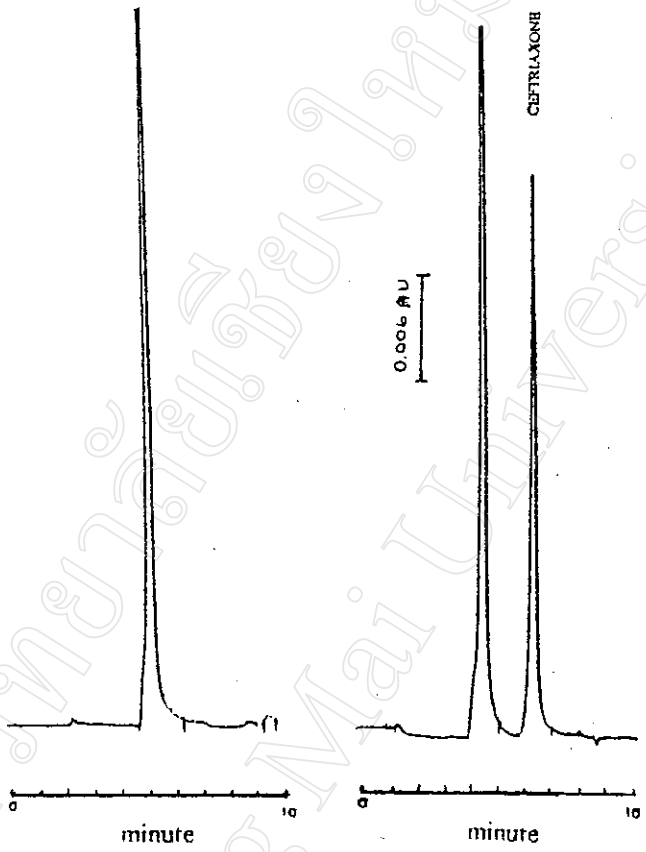
Ceftriaxone is a very polar compound that ionizes at physiologic pH (pK_as of 3, 3.2 and 4.1). Chromatographic retention for polar anionic compounds is limited, but can be enchanced by ion-pairing the drug with quaternary ammonium ions such as HDTA (Ascalone and Dalbo, 1983).

Five different conditions of mobile phases were investigated in this study using the same column before selection of the most appropriate chromatographic condition. The amount of HDTA used in the aqueous phase was 0.182 g% in 0.01 M potassium phosphate buffer. List of solvent compositions (volume ratios) studied and the retention time were :

Buffer pH 8 + acetronitrile (85:15) : retention time 5 min
HDTA in buffer pH 8 + acetronitrile (50:50) : retention time 3-4 min
HDTA in buffer pH 8 + acetronitrile (60:40) : retention time 6-8 min
HDTA in buffer pH 8 + acetronitrile (65:35) : retention time 8-10 min
HDTA in buffer pH 7 +acetronitrile (60:40) : retention time 11-13 min

The mobile phases were stirred throughout the analysis procedure.

The retention time of ceftriaxone was shown to be affected by the composition and condition of the mobile phases. We selected 0.182 g% HDTA in 0.01 M potassium phosphate buffer pH 8 : acetronitrile (60:40, v/v) as the mobile phase in this study since the yielded chromatogram of ceftriaxone was free from any interference peak, with well resolution, sharp peaks and optimal retention time (Fig.3).



(A) (B)

Figure 3. Typical chromatogram of ceftriaxone in the serum after an extraction procedure; (A) before and (B) 1 hr after an intramuscular administration of ceftriaxone.

1.2 Selection of dilution solvent

We used 0.01 M potassium phosphate buffer at pH 8 to dilute the urine samples before an extraction procedure. Dilution of the urine samples with this buffer yielded an interference-free chromatogram by the HPLC assay.

1.3 Validity and precision of the HPLC assay

Intraday and interday variations were evaluated by adding various known amounts of ceftriaxone to the serum. Six samples were analyzed on the same day for six consecutive days. The coefficient of variations of this HPLC method for determining ceftriaxone concentration were less than 11%(Table 4).

Table 4 Precision of the HPLC analytical procedure.

Known ceftriaxone concentrations (ug/mL)	Apparent ceftriaxone concentrations determined by HPLC (ug/mL)			
	intraday value		interday value	
	Mean ± SD	%CV	Mean ± SD	%CV
50 (n=6)	48.2 ± 0.21	4.3	45.2 ± 4.9	10.8
5 (n=6)	4.7 ± 0.12	2.5	4.6 ± 0.32	6.9

Calibration curve plotted as peak areas of known concentrations of ceftriaxone (4-200 ug/mL in 0.01 M potassium phosphate buffer pH 8) was linear with the correlation coefficient of 0.999 (n=12)(Table 5, Fig 4).

Table 5. Peak area of known concentrations of ceftriaxone in buffer determined by the HPLC assay.

Known ceftriaxone concention (ug/mL)	Peak area (millivolts/thousand) mean ± SD
200 (n=3)	1,275,656 ± 189,448.2
100 (n=3)	625,150 ± 79,787.7
20 (n=3)	121,261 ± 13,399.5
4 (n=3)	24,992 ± 1,886.5

1.4 Sensitivity

The sensitivity of this HPLC method is adequate to permit a detection of the drug in a concentration as small as 0.0625 ug/mL.

2. Extraction procedure:

Five hundred uL of serum samples were mixed throughly with 1,000 uL of acetronitrile to precipitate protein. Urine samples were diluted to 1: 5 with 0.01 M potassium phosphate buffer pH 8, and 1,000 uL of acetronitrile was then added to 500 ul of diluted urine sample for deproteinization. Both the serum and urine mixtures were

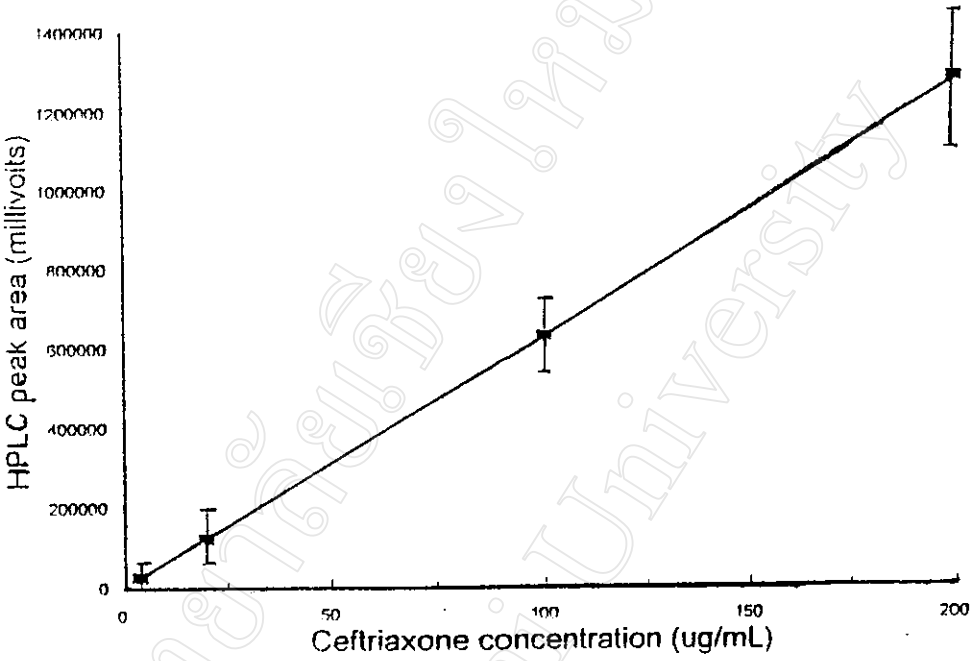


Figure 4. Standard curve for ceftriaxone determination.

vortexed for 1 min and centrifuged for 3 min at 13,000 rpm. A total of 10 uL of the supernatant from both the serum and urine samples were then injected into the HPLC column (Granich and Krogstad, 1986, Jungbluth and Jusko, 1989).

Recoveries of ceftriaxone from serum and urine after the extraction procedure were determined by comparing the peak area of ceftriaxone after the extraction procedure with the peak area of the equivalent standard concentration of ceftriaxone in the buffer. For the serum samples, recoveries were found be 93.4 $\pm$ 19.7 (n=6) and 84.1$\pm$8.8% (n=6)(mean 88.7 $\pm$ 6.6%) for the ceftriaxone concentrations of 50 and 5 ug/mL, respectively. For the urine sample, recoveries were found to be 85.7$\pm$ 2.5 (n=6) and 90.2 $\pm$ 4.6% (n=6) (mean 87.9$\pm$ 3.2%) for the ceftriaxone concentrations of 50 and 20 ug/mL, respectively (Table 6). Concentrations of ceftriaxone in the samples obtained from each subject determined from the standard curve all were corrected for the percent recovery after the extraction procedure to yield true concentration of ceftriaxone in samples. Known concentrations of standard ceftriaxone in the pooled serum were also determined in parallel on each day of analysis to ensure the accuracy of the HPLC assay.

Table 6 Percent recovery of serum and urinary ceftriaxone following the extraction procedure.

Ceftriaxone concentrations (ug/mL)	Apparent ceftriaxone concentration (ug/mL)	% Recovery
Serum 50 (n=6)	46.7 ± 9.8[a]	93.4 ± 19.7
5 (n=6)	4.2 ± 0.4	84.1 ± 8.8
Urine 50 (n=6)	42.8 ± 1.3	85.6 ± 2.5
20 (n=6)	18.0 ± 0.9	90.2 ± 4.6

a Mean ± SD

3. *In vitro* quantitation of ceftriaxone

ROCEPHIN and CEF-3 were reconstituted aseptically in a 3.5 mL of injection solvent which contains 1% lidocaine solution. Aliquots of 500 uL solution and dry powder form of both ROCEPHIN and CEF-3 were stored at -11°C, 0° C, and room temperature (26-30° C) for 1, 4, 9, 30 and 90 days. At the designated time, samples were diluted to 1: 500 with 0.01 M potassium phosphate buffer pH 8 before analysis for ceftriaxone concentrations using the HPLC.

Pharmacokinetic analysis

PCNONLIN

PCNONLIN, the estimated program for statistical analysis of general nonlinear models, was employed to obtain the parameter estimates of the equation decribing the pharmacokinetic profile of

ceftriaxone. The appropriate models used in this study were model 3, one-compartment with first-order input, first order output, and no lag time for drug given by an intramuscular route.

Plasma concentration-time curve of ceftriaxone is adequately characterized by a bi-exponential equation corresponding to a one - compartment open model with first - order absorption and elimination which depicts the body as a single homogenous unit in which rate of distribution over the body and elimination from the various organs are of first order and proportional to drug concentrations. The important pharmacokinetic parameters defining the fate of drug in the body are T_{max}, C_{max}, $t_{1/2}$, AUC, V_d, K_a, K_e, CL_p, renal clearance (CL_r). We also determined the relative bioavailability (F_{rel}), percent unchanged ceftriaxone recovered in the urine over 24 hr, fraction of the dose excreted in urine (f_e), renal excretion rate constant of ceftriaxone (K_r) and nonrenal excretion rate constant (K_{nr}).

The amount of ceftriaxone recovered in the urine over 24 hr was calculated by multiplying the urine volume and the urinary concentration of ceftriaxone measured at intervals; 0-2, 2-4, 4-8, 8-12 and 12-24 hr after an intramuscular of ceftriaxone.

K_r of ceftriaxone was estimated from the following relationship:

$$dAu/dt = K_rA \quad \text{.....(1)}$$

$$A = Aoe^{-k_et}$$

$$dAu/dt = K_rAoe^{-k_et} \quad \text{.....(2)}$$

$$\text{Log } dAu/dt = \text{Log } K_rAo + \text{Log } e^{-k_et} \quad \text{.....(3)}$$

$$\text{Log } dAu/dt = \text{Log } {}_{K_r}Ao - k_et \times \text{Log } e \quad \text{.....(4)}$$

Where Au is amount of drug excreted unchanged in the urine, A is amount of drug administration, t is time at measurement, and Ao is amount of drug at t = 0.

K_e is obtained from the value of the slope of the natural semilogarithmic plot of the urinary excretion rate versus time and Kr is the amount of drug at t=0 (Yo/Ao) which is obtained as the value at y-interception axis. The nonrenal excretion rate constant (K_{nr}) can be determined as $K_{nr} = K_e - K_r$

Renal clearance and the fraction of the unchanged dose in urine were calculated by following equations

$$CL_r = A\ (t_1 \text{ to } t_2) / AUC\ (t_1 \text{ to } t_2)$$

$$f_e = CL_r / CL_p$$

Where A (t_1 to t_2) represents the amount of drug excreted unchanged in the urine from time t_1 to t_2 and AUC (t_1 to t_2) is the area under the plasma concentration-time curve during time interval from t_1 to t_2.

Statistical analysis

The paired Student's *t* test was used to determine the statistical significance of the difference in the mean values of all pharmacokinetic parameters such as T_{max}, C_{max}, AUC, $t_{1/2}$ and the amount of drug excreted in the urine when the same dosage of the two products, ROCEPHIN and CEF-3, were administered. The unpaired Student's *t* test was used to determine the statistical significance of difference in the mean values of all pharmacokinetic parameters when different doses of the two products, ROCEPHIN and CEF-3 were administered. P value of < 0.05 was considered statistically significant.