CHAPTER III

MATERIALS AND METHODS

Materials

A. Test Products

Three commercial brands of ceftriaxone intramuscular injections were randomly purchased from hospitals. Each vial contains ceftriaxone sodium equivalent to 1 g. of ceftriaxone, one was the innovator's product which was assigned as the reference standard.

The letters A, B and C were given to represent the brand names of each product. Other informations of these products were all summarized in Appendix A

B. Reagents

1. Working standard ceftriaxone powder (Siam Pharmaceutical Co., Ltd.)
   potency : 84.71%, Lot No. 045193
2. Working standard ciprofloxacin powder (Siam Pharmaceutical Co., Ltd.)
   potency : 93.61%, Lot No. 04892
3. Acetonitrile HPLC grade (May and Baker, England)
   Lot No. MA 2351
4. Methanol HPLC grade (J.T. Baker Inc., U.S.A.)
   Lot No. G 36318
5. ortho-Phosphoric acid 85% GR. (E-Merck, Germany)
   Lot No. K 15068573
6. Monobasic potassium phosphate AR. (E-Merck, Germany)
   Lot No. A 646173E
7. Dibasic potassium phosphate AR. (Carlo Erba, Italy)
   Lot No. 3 B 662213E

8. Ammonium acetate AR. (Carlo Erba, Italy)
   Lot No. 3E 526033M
9. Glacial acetic acid 100% AR. (J.T.Baker Inc., U.S.A.)
   Lot No. B 25PO260
10. Triethylamine AR. (E-merck, Germany)
    Lot No. 3312778
11. Heparin injection BP (Porcine mucous) 5000 i.u./ml
    Lot No. 3033096 DOM
12. Normal saline for injection 0.9% (General Hospital Products Public)
    Lot No. 32-699-XL
13. 10 N Potassium hydroxide AR. (E-Merck, Germany)
    Lot No. C 624033
14. Chloroform AR. (E-Merck, Germany)
    Lot No. K 20567745

C. Apparatus

1. Analytical balance.(Sartorius, 1615 MP ; S/N 3209026, Germany)
2. Digital pH meter (SA 520, Orion, U.S.A.)
3. Vortex mixer (vortex - Genie, Scientific Industries Inc., U.S.A.)
4. Refrigerated centrifuge (Sigma 302 K, Sigma Lab, Centrifuge Gmbtt, Germany)
5. High performance liquid chromatography
   - Column : μ-Bandapak® (C18, waters, U.S.A.)
   - Integrator : C-R1A File No. 2 (Shimadzu®, Japan)
   - Absorbance detector : Spectro Monitor® 4100
   - HPLC pump : LC-3A (Shimadzu®, Japan)
6. Personal computer (Acer Mate 433)
7. Micropipette (Socorex, Switzerland)
8. Sonicator (Bansonic 221, U.S.A.)
9. Glassware

Methods

A. In Vitro Studies

All of the three commercial brands of ceftriaxone intramuscular injection (IM), 1 g., were evaluated according to the official tests stated in the monograph of ceftriaxone sodium injection in the United States Pharmacopoeia 1995 (U.S.P. XXIII).

1. Uniformity of Dosage Units

Weight variation

Weigh accurately 10 vials individually, taking care to preserve the identity of each vial. Remove the contents of each vial. Weigh accurately the emptied vials individually, and calculate for each vial, the net weight of its contents by subtracting the weight of the vial from the respective gross weight. The average weight was calculated in term of ceftriaxone anhydrous (mg)/ceftriaxone sodium hydrous (g).

2. Assay for content of active ingredient

The amount of each brand of ceftriaxone in vial was determined according to the modified method from that of the U.S.P. XXIII. The method was described as follows :

pH 7.0 buffer : Dissolve 13.6 g. of dibasic potassium phosphate and 4.0 g. of monobasic potassium phosphate in water to obtain 1000 ml. of solution. Adjust this solution with ortho-phosphoric acid or 10 N potassium hydroxide to a pH of 7.0±0.1.

Mobile phase : 90:10 v/v of 0.1M phosphate buffer pH 7.0 and acetonitrile were mixed. Filter through a membrane filter of 0.45 μm., and degas before use.

Standard preparation : Dissolve an accurately weighed quantity of the U.S.P. ceftriaxone sodium working standard in mobile phase, to obtain a solution having a known

concentration of about 50 μg/ml. Use this solution promptly after preparation. (Repeat for three sets of preparation).

Assay preparation : Transfer about 20 mg. of ceftriaxone sodium, accurately weighed, to a 50 ml. volumetric flask, dissolve in mobile phase, dilute with mobile phase to volume, and mix. Pipette 3 ml of solution accurately, to a 25 ml. volumetric flask, dilute with mobile phase to volume. (Repeat for three sets of preparation of each brand).

Chromatographic condition

Apparatus : Shimadzu® LC-3A HPLC pump, equipped with Spectro Monitor® 4100 variable wavelength UV detector and Shimadzu® C-R1A File no. 2 integrator.

Column : μ-Bondapak® (C18), stainless steel column, 3.9x300 mm., 125°A 10 μm. of dimethyloctadecylsilyl boned amorphous silica, part no. 27324, serial no. T 21011k03 (Waters Associates Pty-Ltd., Milford, MA, U.S.A.)

UV detector : 252 nm.

Flow rate : 1.0 ml / min.

Chart speed : 2 mm. / min.

Pressure : 140 kg / cm^2

Attenuation : 2^2 mv / full scale

Operating temperature : ambient

Injection volume : 20 μl

Retention time : ceftriaxone 4 min. (Figure 2)

Procedure : Separetely inject equal volumes of the standard preparation and the assay preparation into the chromatograph, measure the responses for the major peaks. Calculate the quantity, in μg, of ceftriaxone per mg. of the ceftriaxone sodium taken by the formula.

$$\% \text{ Labeled amount} = \frac{A_u}{A_s} \times \frac{C_s}{C_u} \times \frac{\text{Average wt./vial}}{\text{Labeled wt.}} \times 100$$

where A_u = Area under the curve detected from the assay preparation.

A_s = Area under the curve detected from the standard preparation.

C_u = Final concentration of the assay preparation.

C_s = Final concentration of the standard preparation

3. Stability Test

The stability test method for reconstituted ceftriaxone IM was not available in any pharmacopoeias. Hence, in order to study the stability of such products, the extended procedure from that of the assay for content of active ingradient was modified and used. It was described as follow. :

Reconstitute two sets of each brand of ceftriaxone IM by water for injection. (Repeat 3 vials for each brand). Each set of them was kept firmly in the control area at 30°C or 4°C. Withdraw 0.5 ml of each solution and place into test tubes, and then pipette 25 µl of the solution and transfer to a 50 ml volumetric flask, adjust with mobile phase to volume. Inject about 20 µl of each sample into the chromatograph, following the same condition as previously described.

The solutions were sampled at the first day and every seven days interval for one month. Calculate the remaining amount of active ingredient by using calibration curve. The physical characteristic of solutions were also observed such as color change, crystallization, etc.

Calibration curve

Standard solutions with known concentration of ceftriaxone were prepared in mobile phase to produce the concentrations of 5, 10, 20, 40, 80, 120, 160, and 240 µg/ml, respectively. All samples were analyzed by HPLC. The area under the peak of ceftriaxone versus the known ceftriaxone concentrations were fitted to a straight line using linear regression (Appendix C).

4. In Vitro Evaluation

The physical and chemical characteristics of all three brands of ceftriaxone IM were examined and evaluated to determine whether each brand passed the requirements as stated in the U.S.P. XXIII.

The remaining amount of active ingredient in the stability test presented in term of percent relatively to the total amount of active ingredient in the freshly reconstituted products. The remaining amounts of active ingredient among the three brands were determined by one way analysis of variance (ANOVA) and/or L.S.D. at the significant level of $\alpha = 0.05$ (Appendix F).

B. In Vivo Studies

1. Test Products

All three commercial brands of ceftriaxone IM, 1 g., were used in this investigation.

2. Subjects

Twelve Thai male healthy volunteers with average 32.33±9.45 years of age (range 23 to 55 years), average weight of 61.92±14.66 Kg. (range 45 to 100 Kg.) and average height of 166.33±6.51 cm. (range 156 to 178 cm.) (Appendix D). Prior to study, physical examination and clinical laboratory tests were clearly explained to all subjects. They gave witten informed consents before participating the study and they were asked to take no medication, alcoholic preparations and cigarettes for at least one week preceeding the study and during the experimental period.

3. Dose and Drug Administration

A 1 g. of ceftriaxone IM was given to each subject by slow injection into deep muscle in the lateral thigh.

4. Experimental Design

The drug was injected to each subject, following a single-blind, randomized crossover design with one-week washout period (Table 2).

To facilitate blood sampling, an IV catheter with a heparin lock (1:200 v/v of heparin solution 5000 i.u./ml in nornal soline 0.9%) was inserted in the antecubital vein, before dosing and remained in place for about 8 hr. Subsequent blood samples were obtained by venipuncture.

5. Sample Collections

Approximate 5 ml. of blood samples were collected and transfered into centrifuge tubes containing heparin (one drop of 5000 i.u./ml of heparin solution) before the administration of ceftriaxone and at 0.25, 0.5, 1, 1.5, 2, 3, 5, 8, 12 and 24 hr. after dosing. Plasma samples were separated by centrifugation at 3000 rpm., for 10 minutes and they were kept frozen at -20°C until subsequent analysis.

6. Determination of ceftriaxone in Plasma

Plasma ceftriaxone concentrations were determined using the modified high performance liquid chromatographic method described by Demotes-Mainard et al. (1988). The procedure was described as follows. :

Plasma sample 0.5 ml.

- add internal standard solution 0.5 ml. (80 μg/ml of ciprofloxacin in 0.1 M ammonium acetate buffer pH 5) (Appendix B)
- Precipitate protein with acetonitrile 2.5 ml.
- vortex 30 sec., centrifuge (3000 rpm., 3 min.)

Clear supernatant

- extract with chloroform 2.5 ml
- vortex 30 sec., centrifuge (3000 rpm., 3 min.)

Clear supernatant

Inject about 20 μl into HPLC

HPLC Conditions for Plasma Ceftriaxone Analysis

Apparatus : Shimadzu® LC-3A HPLC pump, equipped with Spectro Monitor® 4100 variable wavelength UV detector and Shimadzu® C-R1A File no. 2 integrator

Column : μ-Bondapak® (C18), stainless steel column, 3.9x300 mm., 125°A 10 μm. of dimethyloctadecylsilyl boned amorphous silica, (Waters Associates Pty.-Ltd., Milford, MA, U.S.A.)

UV detector	:	270 nm.
Flow rate	:	2.1 ml / min.
Attenuation	:	2^2 mv / full scale
Pressure	:	220 kg / cm^2
Operating temperature	:	ambient
Chart speed	:	2 mm. / min.
Injection volume	:	20 μl
Retention time	:	ceftriaxone 2.9 min.
		ciprofloxacin 4.9 min. (Figure 3)

Mobile phase : water : methanol : triethylamine (750 : 250 : 4 v/v/v), adjust to pH 3.0±0.1 with ortho-phosphoric acid, filtered and degassed before use.

The concentrations of plasma ceftriaxone samples were quantified from the calibration curve (Appendix C).

7. Calibration Curve

Stock solutions of ceftriaxone and ciprofloxacin (1 mg/ml) were prepared in tripply distilled water (Appendix B). They were stored at -20°C. Appropriate dilutions of ceftriaxone stock solution were made in drug-free human plasma to provide concentrations of 5, 10, 20, 40, 80, 120, 160 and 240 μg/ml. The internal standard concentration (ciprofloxacin) was 80 μg/ml in 0.1 M ammonium acetate buffer (pH 5) (Appendix B). All samples were analyzed following the same procedure aas previously described. Plasma blank was prepared using distilled water instead of ceftriaxone. The ratios of the peak height of ceftriaxone to the

internal standard versus the known ceftriaxone concentrations were fitted to a straight line using linear regression (Appendix C).

8. Assay Validation

The modified Demotes-Mainard et al's method was validated under the following conditions.

8.1 Within-run precision

This precision was determined by analyzing the three sets of the calibration curves at the same day. Peak height ratio of ceftriaxone to ciprofloxacin was compared and the percent coefficient of variation (%C.V.) for each concentration was determined (Table 9)

8.2 Between-run precision

This precision was determined by comparing the three sets of the calibration curves on three different days and the percent coefficient of variation (%C.V.) for each concentration was calculated (Table 10).

8.3 Percent recovery

Determination of percent recovery was done by comparing both the peak height of ceftriaxone and ciprofloxacin spiked into plasma to each of those spiked into mobile phase (Table 11)

9. Pharmacokinetic Analysis

Intramuscularly plasma concentration-time profiles from each subject following administration of all three brands of 1 g. ceftriaxone products were analyzed using the PCNONLIN computer program.

The peak plasma concentration (C_{max}), the time to reach the peak plasma concentration (T_{max}), the area under the concentration-time curve (AUC) and the biological half-life ($t_{1/2}$) of ceftriaxone were directly obtained from the computer outputs.

10. Bioequivalent Evaluation

The bioequivalence of all three brands of ceftriaxone IM were evaluated using the three relevant pharmacokinetic parameters, C_{max}, T_{max}, and AUC.

The differences of these three pharmacokinetic parameters among the three brands were determined by one way analysis of variance (ANOVA) and/or t-test at the significant level of $\alpha = 0.05$.

Table 2 Treatment schedule

Subject Number	Week		
	1	2	3
1	A	B	C
2	B	C	A
3	C	A	B
4	A	B	C
5	B	C	A
6	C	A	B
7	A	B	C
8	B	C	A
9	C	A	B
10	A	B	C
11	B	C	A
12	C	A	B

A, B, C represented the brand name of ceftriaxone intramuscular injection