CHAPTER III

MATERIALS AND METHODS

Materials

1. Test Products

Eight commercial brands of doxycycline, 100 mg capsules, were purchased from drugstores without any attempt to procure or select lots. The letter (A, B, C, D, E, F, G and H) were given to represent the brand names of products. Information of test products were accesible in appendix A

2. Reagents

2.1 Working Standard Doxycycline Hydrochloride Powder, Potency 88.42% Lot no. 880809 (Biolab Co. Ltd.)

2.2 Internal Standard; Tetracycline Hydrochloride Powder, Lot no 89-4184 (Department of Medical Science)

2.3 Monobasic Sodium Phosphate GPR [BDH Chemicals, England] lot no 9528440E

2.4 Methanol P.A. (E. Merck , West Germany) Lot no. K 12345309

2.5 Sodium Hydroxide AR grade (E. Merck, West Germany) Lot no. 735 F 647598

2.6 N, N-Dimethyloctylamine (Aldrich Chemicals, U.S.A.) Lot no. 06813 IV

2.7 Sodium Citrate USP (Vidhyasom) Lot no. 000325

2.8 Acetonitrile AR grade (E. Merck, West Germany) Lot no..

8248408

2.9 Ortho phosphoric Acid 85% P.A. (E Merck, West Germany) Lot no. K 1018973

2.10 Ethyl Acetate AR grade (E. Merck, West Germany) Lot no. K 11698523

2.11 Sodium Sulfite AR grade (E. Merck, West Germany) Lot no. 626 K 2368557

2.12 Concentrated Sulfuric Acid AR grade (E. Merck, West Germany) Lot no. 922K 12474731

2.13 Heparin 5000 IU/ml (Novo Industries, Denmark) Lot no. 3390025

3. Apparatus

3.1 Analytical Balance (Mettler 51 AR)

3.2 Disintegration Tester (GC-21, Hanson Research Corp., Northridge, Calif., USA)

3.3 Dissolution Apparatus (72 RL, Hanson Research Corp., Northridge, Calf., USA)

3.4 Spectrophotometer (Spectronic 2000, Bausch & Lomb, N.Y., USA)

3.5 High Pressure Liquid Chromatography (LC-3A, Shimadzu Japan)

3.6 Centrifuge (GmbH 302K, Sigma, West Germany)

3.7 Digital pH meter (PBS 730, El-Hama Instruments, Israel)

3.8 Vortex mixer (Vortex-Genie, Scentific Industries. Inc., Bohemia, N.Y., U.S.A)

3.9 Waterbath (W-O 350, Willi Memmert KG, West. Germany)

3.10 Digital Computer (IBM Compatible 16 Bit, Micro Source)

3.11 Micropipet (Socorex ®, Switzerland)

Method

1. In Vitro Studies

Eight brands of doxycycline, 100 mg capsules, were evaluated by using the official and non-official test of U.S.P and / or B.P. for capsules.

The tests include:

1.1 Uniformity of Weight B.P. 1988 (39)

Twenty capsules of each of the eight brands of doxycycline capsules were sampled. Each intact capsule was weighed and opened without losing any part of the shell. The contents were brushed out as completely as possible. The separated shell was weighed accurately. The weight of the content is the difference between the two weighings. The procedures were repeated with further nineteen capsules. The average weight and standard deviation were calculated.

1.2 Content of active ingredient

The amount of doxycycline in capsules was determined according to the USP XXI method (40) which was described as follow:

The content of twenty capsules of each brand were removed as completely as possible, and weighed accurately. The combined contents were mixed. An accurately weighed portion of the powder, equivalent to about 50 mg of doxycycline was accurately weighed and transferred to a 50-ml volumetric flask. About 35 ml of water were added and shaken for 15 minutes, water was adjusted to volume. The

solution was then mixed and filtered through filter paper. The first 10 ml of the filtrate was discarded, and the filtrate was filtered through a membrane filter of 0.5 µm or finer porosity. The final filtrate was then assay by HPLC

HPLC conditions for doxycycline analysis in capules

Apparatus	:	HPLC LC-3A, Shimadzu, Japan
Column	:	µ - Bondapak C_{18}, stainless steel column, Water Associates Pty. Ltd., U.S.A. Pre-column 5cm x 2.0 mm i.d. analysis - column 30 cm x 3.9 mm i.d.
Mobile phase	:	450 ml of 0.1 M monobasic sodium phosphate and 550 ml of methanol, 3 ml of N,N - dimethyl-n-octylamine was added and adjusted with 5 N sodium hydroxide to pH 8.0
UV detector	:	280 nm
Flow rate	:	1.5 ml/min
Attenuation	:	2^5 mv/full scale
Pressure	:	230 Kg/cm^2
Temperature	:	ambient
Volume injected	:	5 µl

The actual content of doxycycline in capsule was quantified utilizing a standard solution (appendix C)

1.3 Content Uniformity (41)

The requirements for content uniformity are met if the amount of the active ingredient not less than nine of ten capsules lies within the range of 85.0 percent to 115.0 percent of the label

claim and and no capsule is outside the range of 75.0 percent to 125.0 percent of label claim and the relative standard deviation of the ten capsules is less than or equal to 6.0 percent.

Ten capsules were assayed individually as directed in 1.2 Detail of the criteria for determination of content uniformity were expressed in appendix D

1.4 Disintegration Test

The disintegration test of doxycycline capsules were determined according to the USP XXI method (42). The precedure was described as follow:

A capsule was introduced into each of the six tubes of the basket. A disk was then added to each tubes, and the apparatus was operated using water maintained at 37 ± 2 °C. The time that the apparatus started to move until all of the capsule have disintegrated and pass through the baskets except for fragments from the capsule shell, was disintegration time .The average and standard deviation of disintegration time of each brand were calculated.

1.5 Dissolution Test

According to the USP XXI 4th supplement (40), the dissolution rate of doxycycline capsule was determined by the USP Dissolution Apparatus Type II (paddle).

Nine hundred milliliters of CO_2-free deionized water was placed in the vessel and equilibrated at 37 ± 0.5 °C. A capsule was

introduced into each of the six vessels, the apparatus was then immediately operated and maintained stirring at speed 75 rpm. Five millitres of dissolution medium was taken from each vessel at 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 minutes intervals and added immediately the same quantity of the medium after each sampling to keep the volume of dissolution medium constant during the course of test. The absorbance of the drug dissolved in dissolution medium were measured using spectrophotometer at 276 nm. The amount of the drug dissolved at various time intervals was quantified using the calibration curve.

Calibration curve

Standard solutions with known concentration of doxycycline in dissolution medium were prepared and analysed using spectrophotometer at 276 nm. Absorbance obtained versus known concentrations were fitted to a straight line using linear regression (appendix F).

1.6 In Vitro Evaluation

Physical characteristics of eight brands of doxycycline capsules were examined and evaluated to determine whether each brand passed the general standard of B.P. and/or U.S.P requirement. A one way analysis of variance and t-test (43) were performed to assess the difference of the disintegration times and the dissolution rate constants between the original and the local brands. The correlation coefficient test was determined between the disintegration time and the dissolution rate constants.

2. In Vivo Studies

2.1 Test Products

Four brands of doxycycline capsules with differences in the in vitro dissolution characteristics were selected. Local made doxycycline capsules which pass the pharmacopoeial requirements will be divided into three groups according to their dissolution rate constants as fast, medium and slow dissolved drugs. One brand will be selected from each group to compare with the original product in the in vivo studies.

2.2 Subjects

Twenty healthy male volunteers with the ages ranged from 18 to 24 years participated in this study. A medical history, completely physical examination and standard laboratory screen for individual subject (appendix E) were performed prior to the study to ensure the absence of any significant hepatic, renal disturbance and/or the gastrointestinal tract disorder. No subject has any history of allergy or hypersensitivity to tetracycline group. The method of the study was fully explained to all subjects and all gave their written consent before entering the study. They were allowed to take no medication for at least one week prior to and throughout the study.

2.3 Drug Administration

Subject fasted overnight before their allocated treatment and remained fasting four hours after drug administration. A single dose of 100 mg doxycycline capsule was taken orally with 200 ml of water.

2.4 Experimental Design

The study was conducted in a randomized crossover design. Each subject received the drug in a randomized order with a two-week washout period between each administration as shown in table 1

2.5 Sample Collection

Five millilitres of blood samples were collected from a forearm vien using a disposable syringe with immediately transfer to heparinized tubes. Blood sample was collected before drug administration and at 0.5, 1, 1.5, 2.5, 3.5, 5, 7, 9, 12, 24 and 33 hours after drug administration. The blood was immediately centrifuged at 300 rpm for 5 minutes, the plasma was separated and kept at -10°C until subsequent analysis.

2.6 Determination of Doxycycline in Plasma

Concentrations of doxycycline in plasma were determined using modified high-performance liquid chromatographic method described by Follath, F., Wenk, M., (44) and Leenheer A.P., and Nelis, H., (45).

2.7 Operating Condition

Apparatus	:	HPLC LC-3A, Shimadzu, Japan
Column	:	μ - Bondapak C 18 stainless steel column Water Associates Pty-Ltd., U.S.A. pre-column 5 cm. x 2.0 mm i.d. analysis - column 30 cm. x 3.9 mm i.d.
Mobile phase	:	0.1 M sodium citrate : acetonitrile (70:30) ajust pH = 2.4 with phosphoric acid

Table 1 Treatment Schedule

Subject No.	Week 1	3	5	7
1	A	B	C	D
2	B	C	D	A
3	C	D	A	B
4	D	A	B	C
5	A	B	C	D
6	B	C	D	A
7	C	D	A	B
8	D	A	B	C
9	A	B	C	D
10	B	C	D	A
11	C	D	A	B
12	D	A	B	C
13	A	B	C	D
14	B	C	D	A
15	C	D	A	B
16	D	A	B	C
17	A	B	C	D
18	B	C	D	A
19	C	D	A	B
20	D	A	B	C

A, B, C, and D represented the brand names of doxycycline capsules

UV detector	:	357 nm
Flow rate	:	1.0 ml/min
Attenuation	:	2^1 mv/full scale
Pressure	:	200 Kg/cm^2
Temperature	:	ambient
Retention time	:	tetracycline 3.19 minutes
		doxycycline 4.45 minutes

The procedure was developed as follows:

plasma sample 0.5 ml

- add 50 ml internal standard* [5 µg/ml in mixture of methanol : 0.01 M HCl 9 : 1]
- add 1 ml of 0.1 M Sodium Sulfite buffer pH 6.2
- mix [vortex 10 seconds]
- extract with 2 * 3 ml of ethyl acetate by vortexing 20 seconds and centrifuge 3000 rpm (2.247 × g) for 5 minutes

combine organic phase into second tube

- evaporate under a gentle stream of nitrogen gas

residue

- reconstitute in 200 ml of the chromatographic mobile phase

inject 75 µl sample solution into the HPLC column

* Tetracycline hydrochloride

The doxycycline concentration in plasma samples were quantified employing the calibration curve as shown in appendix F.

2.8 Calibration curve

Known amounts of 0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0 and 1.25 µg of standard doxycycline and 0.25 µg of internal standard were added to 0.5 ml of pooled human plasma to make the concentrations of 0, 0.1, 0.2, 0.5, 1.0, 1.5 and 2.5 µg/ml respectively. These samples were analyzed following the same procedure as described previously.

2.9 Pharmacokinetic Analysis

Individual plasma doxycycline profile from each treatment was analyzed using both noncompartmental and compartmental method

Noncompartmental analysis do not require the assumption of a specific compartment. The following parameters were calculated under the noncompartmental program.

- The area under the plasma level-time curve from zero time to time t , the time when drug sampling is stopped, [AUC_o^t]

- The area under the plasma level-time curve from zero time to infinity, [AUC_o^∞]

- The elimination rate constant , [Ke]

The estimation of the area under the plasma level-time curve from zero time to infinity, AUC , must be carried out in two steps.

The area under the curve from zero time to t, AUC_o^t is calculated according to of the trapezoidal rule. To this partial area we must add the area under the curve from t to infinity which is equal to C_t/Ke [while C_t is the plasma concentration at the last sampling time, Ke is the elimination rate constant]. Hence, the area under the curve from time zero to infinity can be expressed by the following equation:

$$AUC_o^{\infty} = \int_{t=0}^{t=t} C_t . d_t + C_t/K_e$$

The peak plasma concentration [Cpmax] and the time to peak plasma level [Tmax] were obtained by reading directly from the plasma concentration-time curve.

For compartmental method, the CSTRIP program [46] (a fortan IV computer program) was used to estimate the initial polyexponential parameters by stripping or method of residuals [appendix K]. The PCNONLIN nonlinear estimating program by iteration [47] was applied utilizing the initial parameters obtained from the CSTRIP program.

In compartmental analysis it is reasonable to use the fewest number of compartments which can adequately describe the experimental data. One compartment open model with or without lag time was used to analyzed the plasma doxycycline profile from treatment.

From the output of CSTRIP program, the microparameter obtained were : the absorption rate constant [Ka], the elimination rate constant [Ke], the lag time [t_o], and the value of A1, A2. Hence, other parameters were calculated by following equation:

$$Tmax = [1 / (Ka-Ke) \ln (Ka / Ke)] + t_o$$

$$Cp_{max} = A_1 e^{-Ke\,[T_{max} - t_o]} + A_2 e^{-Ka\,[T_{max} - t_o]}$$

$$AUC_o^{\infty} = \frac{A_1}{Ke} + \frac{A_2}{Ka}$$

$$Vd = \frac{Dose}{Ke\,[AUC]}$$

The parameters [Vd, Ka, Ke, t_o] obtained from the CSTRIP program were as the initial estimates for the PCNONLIN nonlinear program. The final estimation of the parameters were obtained by repeatedly entering the computed parameter values as initial estimation until the values were the best fit to the data.

2.10 Statistical Evaluation for Bioavailability Results

The comparative bioavailability of the four brands of doxycycline capsules were evaluated using the following parameters:

a. The area under the plasma concentration-time curve [AUC]
b. The peak plasma concentration [Cpmax]
c. The time of the peak plasma concentration [T_{max}]
d. The first order absorption rate constant [K_a]

A one-way analysis of variance [ANOVA] and Student's t-test were performed to test the differences among the four brands of doxycycline capsules.

3. In Vitro - In Vivo Correlation Study

The relationship between the in vitro and the in vivo parameters was analyzed using correlation coefficient test, then t-test was performed to test whether or not correlation was statistically significant. The interesting in vitro parameters were the disintegration time and the dissolution rate constant while the in vivo parameters selected to study were those parameters which showed significantly different among brands. The parameters were the absorption rate constant [Ka], the area under the plasma concentration-time curve [AUC], the peak plasma concentration [Cpmax] and the time to peak plasma level [T_{max}].