ASSESSMENT OF BIOEQUIVALENCE OF CIPROFLOXACIN IN HEALTHY MALE SUBJECTS USING HPLC

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ABSTRACT
Pharmacokinetics and bioequivalence studies of two brands of ciprofloxacin 500mg *(Ciprofloxacin & Ciproxin)* were evaluated in 14 healthy male volunteers after oral administration. The drug was analyzed in plasma samples by using HPLC. The peak plasma concentrations of (2.28±0.04 mg/L) and (1.9±0.02 mg/L) was attained in about 1.71 hours and 2.00 hours for both Test and Reference ciprofloxacin, respectively. The mean ± SE values for total area under the curve (AUC) were 11.91±0.26 and 13.11±0.27 h.mg/L for both test and reference tablets respectively. This study indicated that all the differences in bioequivalence parameters for both ciprofloxacin formulations are statistically non-significant, hence both formulations are bioequivalent.

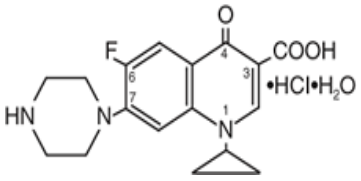
Keywords: Ciprofloxacin two different formulations, area under the curve, peak plasma level, time at which maximum concentration of drug.

INTRODUCTION

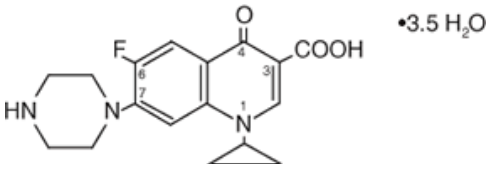
The pathogenic bacterial born diseases remain a constant threat to human's health throughout the world. The problems are more prevalent in developing countries because of poor hygienic conditions, lack of education and financial resources. Prevention of these diseases is a consistent endeavor to enhance the quality of health and life. Antibiotics play a pivotal role to check these diseases and are one of the extensively used drugs throughout the world but more so in the developing countries. Development of antibiotic resistance in bacteria continuously incites the scientists to modify the existing drugs or to develop newer remedies.

Fluoroquinolones are intended for use by the infectious disease specialists, general practitioners, pharmacologists, pharmacists, advance nurse clinicians, nurse practitioners, and other physicians involved in the treatment of patients with respiratory and other infections. Fluoroquinolones have become an extremely important addition to our armamentarium against infection. The role of the newest generation of fluoroquinolones, released in the late 1990s, is still being developed. The addition of the fluorine atom improves potency, enhances antimicrobial activity, and alters pharmacokinetics properties, which provide tremendous therapeutic advantages. During the 1980s, fluorinated derivatives of quinolones were introduced (Ciprofloxacin, 1987) and are now freely available in Pakistan. These fluoroquinolones are useful for treating infections caused by some gram positive and gram negative organisms because pharmacokinetic characteristics render them suitable for treating systemic infections.

In the market combination of two types of ciprofloxacin



drug substance are available, ciprofloxacin hydrochloride and ciprofloxacin betaine (base). Ciprofloxacin hydrochloride is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid hydro-chloride. It is provided as a mixture of the monohydrate and the sesquihydrate. The empirical formula of the monohydrate is $C_{17}H_{18}FN_3O_3$ • HCl • H_2O and its molecular weight is 385.8. The empirical formula of the sesquihydrate is $C_{17}H_{18}FN_3O_3$ • HCl • 1.5 H_2O and its molecular weight is 394.8. The drug substance is a faintly yellowish to light yellow crystalline substance. The chemical structure of the monohydrate is as follows: Ciprofloxacin betaine is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. As a hydrate, its empirical formula is $C_{17}H_{18}FN_3O_3$ • 3.5 H_2O and its molecular weight is 394.3. It is a pale yellowish to light yellow crystalline substance and its chemical structure is as follows:



Ciprofloxacin a zwitterions has good penetration and accumulation in tissues with a wide distribution throughout the body. One of the most impressive properties of Ciprofloxacin is its ability to exert a very rapid bactericidal effect on bacteria. Within 5-10 minutes of Ciprofloxacin being added to the culture medium, the number of organisms falls dramatically. Unlike penicillin

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and cephalosporin, Ciprofloxacin shows equal bactericidal activity during the resting and reproductive phase of bacteria. This would explain the rapid onset of action of Ciprofloxacin and its potency (Zeiler and Grohe, 1984).

Absorption and disposition kinetics studies are important to compare the rate and extent of systemic absorption of a drug manufactured by different manufacturers. Variations in excepients and manufacturing process can affect the disintegration and dissolution rate of tablets given through the oral route. Since, local population shows distinct nutritional habits and thrives in particular environments; therefore, there is a likelihood of differences in biodisposition of Ciprofloxacin. Recently, Seth *et al.* (1995) recorded disposition kinetics of Ciprofloxacin and suggested the need to be cautious while treating patients with renal problems and proposed to use lower doses in Indian patients to achieve desirable results. Therefore, it is always advisable to perform disposition kinetics and renal handling studies in the target population and environments.

The term "bioavailability" refers to the rate and extent to which a drug/nutrient reaches its site of action or a biological fluid such as blood that has access to its site of action. The term "bioequivalence" refers to pharmaceutically equivalent drug products where the rates/extents of bioavailability of the active ingredients are not significantly different under suitable test conditions. In other words, this is a comparison of two or more products with respect to their bioavailability. Bioequivalent simply means that one brand or dosage form of a drug or supplement is equivalent to a reference brand or dosage form of the same drug or supplement in terms of various bioavailability parameters (FDA's regulation 21.CFR-320.1 1991).

In view of the importance of the process of drug absorption as a direct determinant of drug efficacy and safety, and since bioavailability determination has not yet been adopted by official compendia as an efficacy indicating test in Pakistan, the present study has been conducted.

The aim of this investigation was to study the bioequivalence of locally manufactured ciprofloxacin tablets in healthy male subjects.

MATERIALS AND METHODS

The study was conducted in accordance with good clinical practice guidelines. Male volunteers (n =14) enrolled for this study were apprised in details about all aspects of the study in easy understandable language and terminologies. Those who agreed voluntarily were registered for further studies. More than 19 years of age, only healthy non-smoker male subjects with homogenous age and body weight were enrolled for the study. Each volunteer signed the "Informed Consent Form" at the time of registration.

Drugs information

Test drug: Ciprofloxacin 500 mg Tablets
B. No. Test
Mfg. Date 01-2002
Exp. Date 12-2005
Bryon Pharmaceuticals (Pvt.) Ltd. 48-A Industrial Estate, Hayatabad, Peshawar, Pakistan

Reference drug: *Ciproxin*[9] 500 mg Tablets
Ciprofloxacin 500 mg
Batch No. 132-B
Mfg. Date 05-2001
Exp. Date 04-2006
Manufactured by: Bayer Pakistan (Pvt.) Ltd., C-21, S.I.T.E, Karachi, Pakistan.

After an overnight fast of at least 8-12 hours, subjects were randomized to receive a single dose of 500-mg Ciprofloxacin reference or test tablet with 250 ml of water. The volunteers were randomly divided into 2 groups of 14 subjects in each group. A replicated-crossover design for the bioequivalence studies with two formulations was used. A seven day washout period was provided between dosing of test and reference tablets.

Sample collection and handling

Before drug administration, a control/blank venous blood sample was collected from each volunteer through a sterile venous Branula 18G (J Vasocan® Branule®, B. Braun Melsungen AG, Malyasa). Following drug administration, serial blood samples were drawn at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, and 12 hours in heparinized centrifuge tubes specially prepared for this purpose. These tubes were chilled and centrifuged under refrigeration for 15 minutes at approximately 2000 rpm. The plasma was separated and stored at -20 °C until analysis.

Demographic and clinical data

The age, weight, height, blood pressure (Systolic/ Diastolic), temperature and body surface area (BSA) of each volunteer was recorded. The Body surface area was calculated with the following formula as used by Hue *el al.,* (2003):

BSA - (W 0.425 x H 0.725) x 0.007184 Where, 'W stands for weight (Kg) and 'H' stands for height (M).

The clinical data including Glucose, Blood urea, Serum Creatinine, Cholesterol, Bilirubin total, SGPT, SCOT, and CPK of all the volunteers was also determined to check the health status of individuals under study.

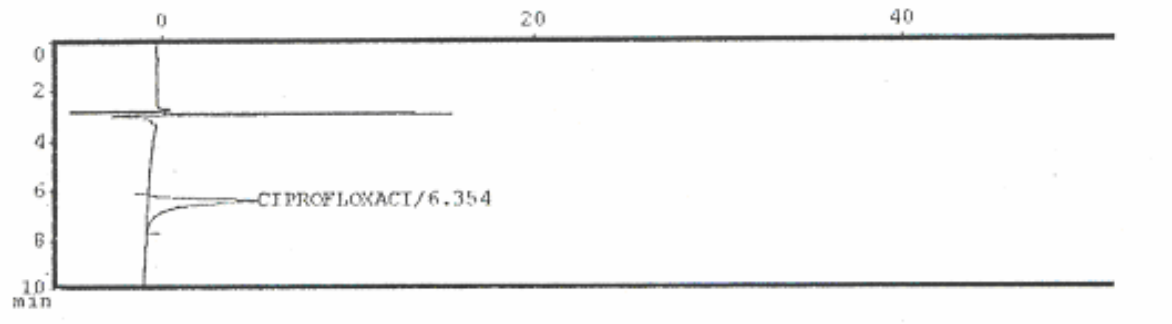
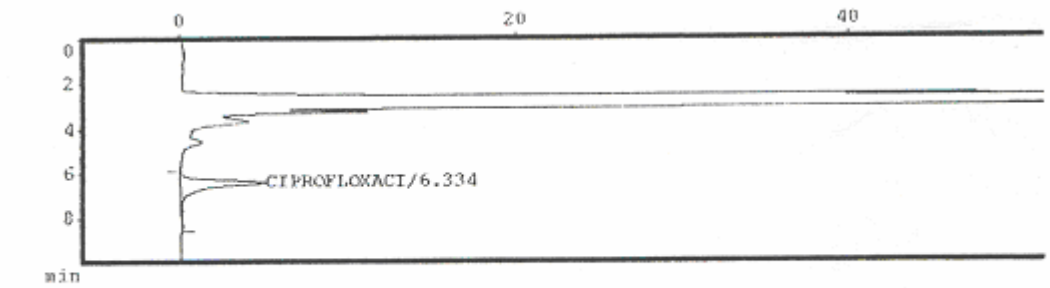


Fig. 1: Standard Chromatogram of 0.5ppm concentration ciprofloxacin.



*** Peak Report ***

PKNO	TIME	AREA	HEIGHT	MK	IDNO	NAME	CONC [%]
1	6.334	113425	5072	S	1	CIPROFLOXACI	72.4069

Fig. 2: Standard plasma spike chromatogram of 0.5ppm concentration ciprofloxacin.

1. High Performance Liquid Chromatography Method (HPLC)

The modified HPLC method was employed for the assay of Ciprofloxacin (Bryon) and standard Ciproxin (Bayer) in the plasma samples.

a) *Instrumentation*

HPLC separation was performed using a (Shimadzu Corporation Tokyo, Japan). LC-10As, Liquid Pump single plunger pump, CDQR system with flow rate range 0.01-5 ml/min (0-400 kgf/cm^2) and 5.01-99.9 ml/min (10-200 kgf/cm^2) with timer program control of flow rate. SPD-10AV-UV-VIS spectrophotometer detector wave-length 190-900nm with range 0.0001 -2.56 AUFS. (285nm) Auto-sampler SIL-10A, Auto injector with samples rack and 1.5 ml sample vial/cap. DATA processor, C-R7A, chromatopac, with 40MB hard Disc Drive, thermal chart paper. The mobile phase contained N-N-Dimethylformamide (6%v/v), Sodium dihydrogen phosphate monobasic (NaH_2PO_4) (0.01 M) Merck, Acetonitrile (15% v/v) HPLC grade, Phosphoric Acid and Distilled water. (6:79:15) P_H=3.0

b) *Material and reagents*

Analytical grade potassium dihydrogen phosphate, acetonitrile HPLC grade (Merck), N,N-dimethyl formamide and phosphoric acid were purchased from the local market. The de-ionized water was purified in the Lab. The pH of mobile phase was adjusted by adding phosphoric acid (85%). Dissolved gasses were removed by 10 minutes sonication on an ultrasonic bath.

c) *Standard solutions*

Standard solutions for plasma determination of Ciprofloxacin were prepared by dissolving Ciprofloxacin in acetonitrile (1mg/ml). Serial dilutions were then prepared in fresh double distilled water (0.2, 0.5, 0.8, 1, 1.5, 2&3 ppm). Blank plasma (1 ml) was spiked with different amounts of standard solutions in order to prepare plasma standards at the time of analysis.

d) *Sample preparation*

Heparinized blank blood samples from same volunteers were centrifuged and the plasma was separated and stored at -20^{O}C until the day of analysis. The frozen plasma was allowed to thaw at room temperature just before the extraction procedure; it was then mixed and centrifuged at 3000 rpm for 15 minutes.

One ml of plasma sample was transferred into a test tube, 0.5 ml of different concentration of ciprofloxacin standard preparations was added and to it 1ml of acetonitrile was

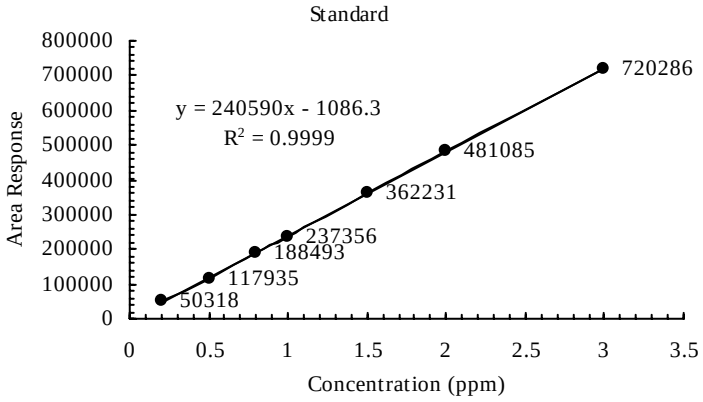


Fig. 3: Standard ciprofloxacin different concentrations calibration curve.

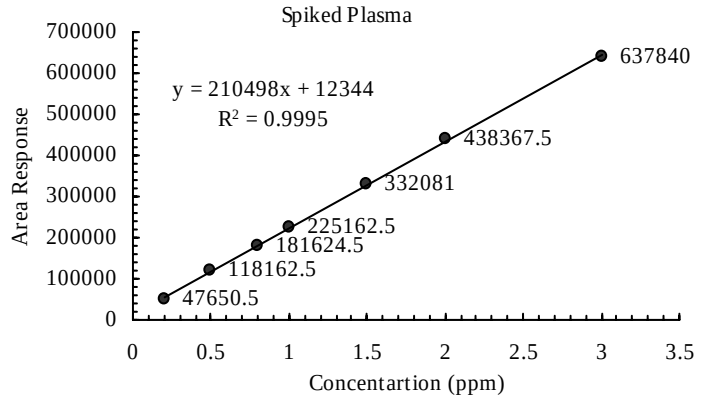
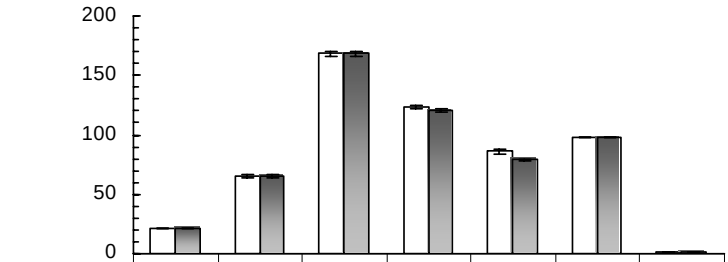


Fig. 4: Standard ciprofloxacin different concentrations spiked plasma calibration curve.



	Age Years	Weight Kg	Height Cm	Syst mmHg	Diast mmHg	Temp oF	BSA m2
Test	21.1	65.9	168.2	123.2	86.1	97.9	1.75
Reference	21.1	65.9	168.2	120.3	79.0	98.2	1.75

Fig. 5: Demographic description of all male volunteers.

than added and the mixture was vortexes for 5 minutes; then the samples were centrifuged and the supernated solution was transferred to HPLC vials for chromatographic analysis, detection at 285nm by using U.V. absorbance.

Chromatography

Aliquots (20µl) of ciprofloxacin reference standard and plasma spiked samples, were injected on to the water micro bondapack C18 column at 50ºC, with a flow rate of 1 ml/minute, with run time 10 minutes. The mobile phase composition Acetonitrile: N,N- Dimethyl formamide: 0.01 M Sodium Dihydrogen Phosphate Dihydrate (15:6:79) and pH3.0 is adjusted by adding phosphoric Acid (85%). The normal chromatograms of ciprofloxacin standards with average retention time of 0.5ppm concentrations was 6.33 minutes at 285 nm U.V absorbance at fig. 1. Ciprofloxacin spiked plasma with

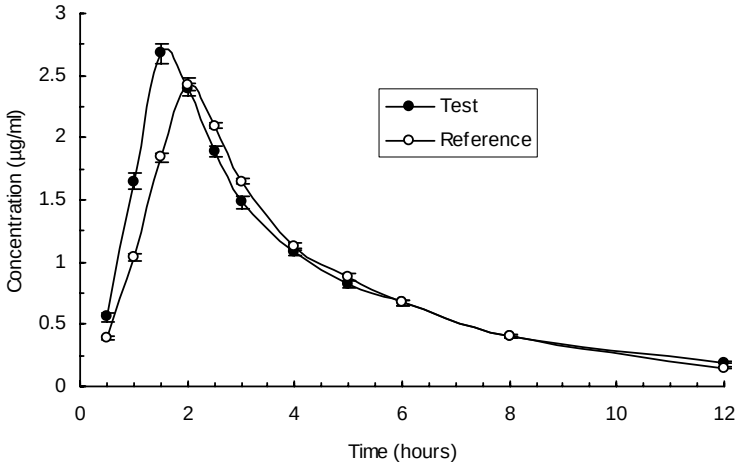


Fig. 6: Time vs. concentration of both test and reference linear plots.

Table 1: Plasma concentration of Ciprofloxacin after an oral dose of 500 mg test tablet (Bryon Pharmaceuticals, Peshawar) and reference tablet Ciproxin® (Bayer Pakistan Pvt. Ltd., Karachi) measured by high pressure liquid chromatography in healthy male volunteers.

Time Hours	0.5	1	1.5	2	2.5	3	4	5	6	8	12
Test Mean ± SE	0.56 ± 0.04	1.65 ± 0.07	2.68 ± 0.08	2.39 ± 0.05	1.89 ± 0.05	1.48 ± 0.05	1.08 ± 0.03	0.82 ± 0.03	0.67 ± 0.02	0.40 ± 0.01	0.19 ± 0.01
Reference Mean ± SE	0.39± 0.02	1.04 ± 0.03	1.84 ± 0.03	2.43 ± 0.05	2.10 ± 0.02	1.65 ± 0.03	1.13 ± 0.03	0.88 ± 0.03	0.68 ± 0.02	0.40 ± 0.02	0.15 ± 0.01
Ratio T/R	1.43	1.59	1.46	0.98	0.90	0.90	0.96	0.93	0.99	0.99	1.30

Table 2: Statistical comparison of bioequivalence parameters for test and reference tablets of 500 mg ciprofloxacin

Bioequivalence parameters	Units	Test Mean ± SE	Reference Mean ± SE	P (T<=t) One -tail	P (T<=t) Two -tail
AUC	[h.mg/L]	11.19 ± 0.22	10.4 ± 0.26	0.0065	0.0151
Ln(AUC)	h.mg/L]	2.41 ± 0.02	2.34 ± 0.02	0.0066	0.0133
Tmax	[h]	1.71 ± 0.02	2.00 ± 0.04	0.0000	0.0000
Cmax	[mg/L]	2.28 ± 0.04	1.90 ± 0.02	0.0000	0.0000
Ln(Cmax)	[mg/L]	0.82 ± 0.02	0.64 ± 0.01	0.0000	0.0000

t Critical one –tail= 1.7709, t critical two-tail = 2.1604 (P>0.05)

average retention time of 0.5ppm concentrations was 6.35 minutes at fig. 2. The calibration curve was constructed using Ciprofloxacin standard sample and Ciprofloxacin spiked plasma samples regression coefficient (R=0.999) and (R=0.995) respectively figs. 3 and 4 (Khan *et al.*, 2005).

b) *Bioequivalence/Bioavailability parameters*

For computation and analysis of Ciprofloxacin in plasma, the computer software programmed "Microsoft Excel 7.0" was used. The plasma concentration of Ciprofloxacin from each volunteer was plotted on linear plot against time. The plasma concentration versus time data was used to calculate pharmacokinetics and bioavailability parameters with the help of a PC-Computer Program, APO, MWPHARM version 3.02 a MEDIWARE product Holland. Calculations also included area under curve (AUC) from time t to infinity (t - infinity) calculated with polyexponential and trapezoidal methods.

(Bioavailability parameters such as C_{max}, T_{max} and AUC were determined) Bioequivalence comparisons were performed using Student t-test: paired two samples for means. For the ratios of the mean bioavailability

Table 3: Mean±SE critical Bioequivalence metrics comparison of bioavailability (bioequivalence) for test and reference tablets of 500 mg.

Bioequivalence parameters	Units	Test Mean ± SE	Reference Mean ± SE	Test % of Reference	90% CI Limits	
					Lower	Upper
AUC*	h.mg/L	13.11±0.27	11.91 ± 0.26	110.0 8	101.91	112.87
Ln(AUC)*	h.mg/L	2.57 ± 0.02	2.47 ± 0.02	104.05	101.31	104.82
T_{max}*	[h]	1.71 ± 0.02	2.00 ± 0.04	85.59	81.78	90.58
C_{max}*	[mg/L]	2.28 ± 0.04	1.9 ± 0.02	120.45	115.71	123.87
Ln(C_{max})*	[mg/L]	0.82 ± 0.02	0.64 ± 0.01	128.91	122.01	135.13

Critical Bioequivalence metrics that should not differ beyond 80-125% except Ln(C_{max}).
Assumption Bioavailability F=1

parameters, models were used to construct 90% confidence intervals for test versus reference tablet.

RESULTS

The demographic data of individuals (Males) who participated in the study of pharmacokinetic/ bioequivalence of Ciprofloxacin Test and Reference formulations is presented in fig. 5. This is evident from the data that volunteers in both study groups are homogenous in terms of mean ± SE age *(*21.1±0.42 years), weight (65.9±1.42 kg), height (168.2±1.89 cm), and body surface area (BSA) (1.73±0.03 m^2). In table 1 Composite plasma drug concentration-versus-time profiles collected from study individuals following oral administration of Ciprofloxacin are presented in fig. 6 on linear plot. The peak plasma concentrations of (2.28±0.15 ug/mL) and (1.90±0.07 ug/mL) was attained in about 1.71 and 2 hours for both Test and Reference Ciprofloxacin respectively. Mean pharmacokinetic data for both Ciprofloxacin preparations are presented in table 1. The mean±SE values for total area under the curve (AUC) were 11.09±0.79 and 10.44±0.93 h. mg/L for both test and reference tablets respectively.

The comparison of mean±SE "bioequivalence" Parameters of Ciprofloxacin Test and Reference formulations have been presented in table 2, while table 3 presents critical bioequivalence metrics comparison for both Test and Reference ciprofloxacin. Statistical appraisal of the bioequivalence between the Unavailability parameters of two formulations did not reveal any significant differences.

DISCUSSION

Ciprofloxacin has become an extremely popular antimicrobial agent for use in human (Owens *et al.*, 1997), dogs, cats, pigs, cattle and poultry (Brown, 1996). The availability of this important drug in various brands in Pakistan raises the need to conduct pharmacokinetic and bioequivalence studies for various formulations in target population. The present project was undertaken to investigate the disposition and bioequivalence of two orally administered formulations of ciprofloxacin. The systemic absorption of an orally administered drug in a solid dosage form is comprised of three distinct steps that can significantly affect the pharmacokinetic and bioequivalence parameters:

1. Disintegration of the drug product.
2. Dissolution of the drug in the fluids at the absorption site.
3. Transfer of drug molecule across the membrane lining the gastrointestinal tract into the systemic circulation.

There is controversy in literature regarding selection of suitable compartmental model to best describe the disposition of ciprofloxacin. The kinetics of ciprofloxacin in domestic animals was mainly described with two compartment open model, as reported in chickens (Anadon *et al.,* 1995), horses (Garcia Ovando *et al.,* 1996), pigs and bovines (Nouws *et al.,* 1988) and ponies (Dowling *et al.,* 1995).

Bioequivalence is a comparison of the Bioavailability of two or more drug products. The two products or formulations containing the same active ingredient are bioequivalent if there rates and extents of absorption are the same. For bioequivalence studies, Cmax, Tmax and AUC are commonly used parameters (table 2). After oral administration of ciprofloxacin, the mean peak plasma concentrations (C_{max}) of 2.28±0.15 ug/mL and 1.90±0.07 ug/mL were attained in about 1.71 and 2.0 hours (T_{max}) for both Test and Reference tablets respectively fig. 6. These values are comparable to the literature values of 3.9± 1.7 mg/L (Catchpole *et al.*, 1994) and between 1.5 to 2.9 ug/mL (Lebel, 1998) after a single 500 mg oral dose. In present study the mean values of area under the curve

(AUC) were 11.19±0.79 and 10.40±0.93 h.mg/L for test and reference ciprofloxacin formulations. This parameter is lesser to the reported values of 20.7±16.6 ug.h/mL (Garrelts *et al.,* 1996) and similar to 12.11 mg.h/L (Escobar and Hoyo, 2003).

It has been reported that the time at which plasma or biological fluid concentrations of the antibiotic exceeds the minimum inhibitory concentration (MIC) is highly correlated with the success of therapy for antibacterial agents exhibiting time-dependent activity (Rao *et al;* 2002). Previous studies suggest that fluoroquinolones kill bacteria in a concentration-dependent manner and area under inhibitory curve (AUIC) calculated by AUC/MC is highly correlated with the outcome of successful treatment (Drusano *et al.,* 1993, Aliabadi and Lees, 1997). For effective eradication of bacteria and good clinical therapy, it has been suggested that an AUIC >100 is required for gram-negative bacteria and >30 is needed for gram-positive organisms (Nightingale *et al.,* 2000; Walker, 2000). Although MIC values of ciprofloxacin for many pathogens of genus Pasturella, Escherichia, Haemophilus, Moraexella, and Salmonella are reported to be in the range of 0.01-0.06 ug/mL (Prescott and Yielding, 1990; Bottner *et al.,* 1995). On the basis of MIC reported for highly sensitive pathogens (0.01-0.06 ug/mL) and AUC (11.19±0.79 and 10.40±0.93 h.mg/L) determined in the present study, AUIC would be much greater than 100.

In the present study the critical bioequivalence parameters included AUC, T_{max} and C_{max} of both test and reference ciprofloxacin are within the range of 80 to 125% (table 3). This study concludes that the bioequivalence metrics between the Bioavailability parameters of both ciprofloxacin formulations did not show significant differences; hence both test and formulations are bioequivalent.

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