

**PHARMACOKINETICS OF NORFLOXACIN AFTER SINGLE
PER-ORAL ADMINISTRATION AND INTERACTION FOLLOWING
CURCUMIN (*Curcuma longa* L.) PRETREATMENT IN RABBITS**

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PAVITHRA B.H

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Dedicated to:

My Beloved Parents...

DEPARTMENT OF VETERINARY PHARMACOLOGY AND TOXICOLOGY
VETERINARY COLLEGE, HEBBAL, BANGALORE
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CERTIFICATE

This is to certify that the thesis entitled 'PHARMACOKINETICS OF NORFLOXACIN AFTER SINGLE PER-ORAL ADMINISTRATION AND INTERACTION FOLLOWING CURCUMIN (Curcuma longa L.) PRETREATMENT IN RABBITS' submitted by Kumari Pavithra B.H, MVHK 618, for the award of the degree of Master of Veterinary Science in Veterinary Pharmacology and Toxicology to the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a bonafide record of research work done by her during the period of her study in this University under my guidance and supervision, and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles

Bangalore,

July 19th, 2008

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LIST OF ABBREVIATIONS

α	Distribution rate constant
β	Elimination rate constant
%	Per cent
@	At the rate of
A°	Armstrong unit
AUC	Area under the curve
AUMC	Area under the first moment curve
°C	Degree centigrade
C _{max}	Maximum plasma concentration
CV	Coefficient of variation
Cl _B	Total body clearance
CYP 3A4	Cytochrome P ₄₅₀ 3A4
CYP 1A1	Cytochrome P ₄₅₀ 1A1
Fig.	Figure
h	Hour
HPLC	High performance liquid chromatography
i.m.	Intramuscular
i.v.	Intravenous
kg	Kilogram
mg	Milligram
ml	Milliliter
min	Minute
MIC	Minimum inhibitory concentration
MRT	Mean residence time
PAE	Post-antibiotic effect
P-gp	P-glycoprotein
pH	$-\log [H^+]$ /negative log of hydrogen ion concentration
p.o	Per oral

rpm	Rotations per minute
R^2	Regression determinant
s.c	Sub cutaneous
SE	Standard error
$t_{\max}$	Time of maximum concentration of drug in plasma
$t_{1/2\alpha}$	Distribution half-life
$t_{1/2\beta}$	Elimination half-life
$t_{1/2ka}$	Absorption half-life
t_d	Total duration of pharmacological effect
μ	Micron
μg	Microgram
$V_{d(\text{area})}$	Apparent volume of distribution (area)
V_{dss}	Volume of distribution at steady state

INTRODUCTION

INTRODUCTION

Antimicrobial agents are among the most dramatic examples of the advances of modern medicine. Antimicrobial therapy exploits differences in structure or biochemical function between host and microbes achieving selective toxicity, and therefore acting as 'magic bullets' in the fight against infectious agents.

The development of fluoroquinolones in early 1980s was a major breakthrough in the treatment of infectious diseases in man and animals. At present, fluoroquinolones, a synthetic class of antimicrobial agents are being extensively used in veterinary clinics including aquatic (fish), exotic pets and wild species worldwide (Prescott and Baggot, 1993).

The fluoroquinolone class of antimicrobial agents are getting widespread acceptance in clinics because of broad spectrum of bactericidal activity against most of Gram positive and Gram-negative aerobes, *Mycoplasma* spp. and *Rickettsia* spp. at very low concentration (Brown, 1996). Additionally, fluoroquinolone group of antibacterial agents offer favourable pharmacokinetic properties *viz*: good per-oral bioavailability, low protein binding, high plasma volume of distribution and low host toxicity (Wolfson and Hooper, 1985).

Norfloxacin is a second generation fluoroquinolone and is primarily used in the treatment of urinary tract infections. However later clinical studies have documented the efficacy of norfloxacin for a variety of Gram-negative infections including pneumonia, CNS/CSF infections, proctitis and septicaemia (Mascellino *et al.*, 1988). Norfloxacin usage in the treatment of gastrointestinal infections caused by *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. were also reported (Braunwald *et al.*, 1987).

Pharmacokinetic properties of fluoroquinolones including norfloxacin vary both between and within species (Neuman, 1987). Pharmacokinetic parameters for norfloxacin have been established in farm and pet animals, but studies in laboratory animals are limited (Gilfillan *et al.*, 1984).

Piperine, an alkaloid naturally found in plants belonging to *Piperaceae* family was reported to inhibit non-specific drug metabolizing enzymes (Annamalai and Manavalan, 1989) and thereby increases the bioavailability of therapeutic agents. Ramesh (2001) observed increased bioavailability of enrofloxacin and its metabolite ciprofloxacin after co-administration of piperine in goats. Similarly, piperine pre-treatment was reported to increase bioavailability of β -lactam antibiotics in rats (Hiwale *et al.*, 2002) and oxytetracycline in hens (Singh *et al.*, 2005).

Similarly, of late curcumin, a flavonoid present in rhizomes of turmeric plant (*Curcuma longa*) was reported to inhibit catalase and UDP-glucuronyl transferase isoenzymes in hepatic and gastrointestinal tract and thereby increasing the bioavailability of mylophenolic acid, an immunosuppressive agent (Owens *et al.*, 2004). Curcumin was also reported to influence P-glycoprotein (P-gp), a protein that regulates drug absorption from gastrointestinal tract in rats (Wenxia *et al.*, 2006)

Therefore, there exists a wide scope to exploit the bioenhancer properties of curcumin in therapeutics so as to increase the bioavailability of drugs. Further, modification of drug metabolism of a given therapeutic agent by curcumin can be exploited clinically so as to manipulate the dose rate and or frequency of administration of drugs. Thus, adverse effect(s) or toxicity of therapeutic agent(s) in question can be minimized or avoided by pre-treatment or concomitant administration of such plant derived active principles.

However, extensive perusal of literature did not reveal studies on pharmacodynamic and or pharmacokinetic interaction between norfloxacin or any other antimicrobial agent with curcumin and or curcuminoids, active principle(s) present in turmeric plant (*Curcuma longa* L.). Keeping these points in view, the present study was undertaken in rabbits with the following objectives:

- a. To determine the pharmacokinetic profile of norfloxacin after single per-oral administration in rabbits
- b. To construct a suitable per oral dosage regimen of norfloxacin for rabbits
- c. To study the influence of curcumin pre-treatment on disposition kinetics of norfloxacin
- d. To derive pharmacokinetic – pharmacodynamic (PK-PD) interactions between norfloxacin and common pathogens infecting rabbits.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

2.1 Fluoroquinolones

The fluoroquinolones have a basic structure of quinolone carboxylic acid. They act by selective inhibition of bacterial DNA *gyrase* enzyme. DNA *gyrase*, a topoisomerase II, consists of 'A' and 'B' subunits. The target site for fluoroquinolones is the 'A' subunit of DNA *gyrase*. Mammals are resistant to the bactericidal effects of quinolone antimicrobials because topoisomerase II in mammalian cells is not inhibited until the drug concentration reaches 100-1000 µg/ml. Fluoroquinolones inhibit most of the pathogenic bacterial multiplication at a concentration less than 0.1 – 10 µg/ml (Papich and Riviere, 2001).

Clinically, fluoroquinolones are preferred in the treatment of urinary tract infections by virtue of its activity against Gram-negative bacteria. However, the new generation fluoroquinolones are recommended against a variety of pathogens causing gastrointestinal, respiratory, skin, bone infections and sexually transmitted diseases (Walker and Wright, 1991).

The fluoroquinolones are active against a wide range of microorganisms. Many bacterial isolates that are resistant to aminoglycosides, third generation cephalosporins and extended spectrum penicillins remain highly susceptible to fluoroquinolones (Neer, 1988). In general, they are most effective against aerobic Gram-negative bacteria followed by Gram-positive bacteria, *Mycobacteria* spp., *Chlamydia* spp. and *Mycoplasma* spp. (Sharma *et al.*, 1994). Several newer fluoroquinolones (often called second generation fluoroquinolones) with increased activity against Gram-positive cocci and anaerobic bacteria includes sparfloxacin, levofloxacin, grepafloxacin and gatifloxacin (Goldstein *et al.*, 1998). All fluoroquinolones are bactericidal

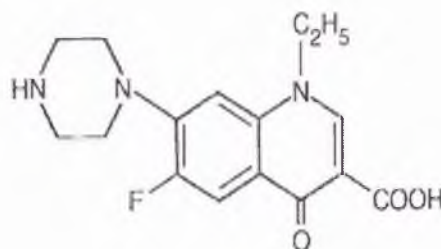
and this activity is maximally seen at specified therapeutic plasma concentration as well as under alkaline environment.

Generally, fluoroquinolones are well absorbed following *per os* administration in monogastrics but absorption is poor in ruminants (Prescott and Baggot, 1993). They penetrate nearly every tissue and cell in the body and therefore possess a large plasma volume of distribution (V_d) and extended plasma elimination half-lives [$t_{1/2\beta}$] (Papich and Riviere, 2001). At appropriate concentration:MIC ratio, the fluoroquinolones are rapidly bactericidal and exhibit a prolonged post-antibiotic effect (PAE) *in vivo* on certain bacteria (Prescott and Baggot, 1993). Often, tissue concentrations of fluoroquinolones are higher than the concurrent serum concentrations by virtue of their large V_d and low plasma protein binding (Orisini and Perkons, 1992). Routinely used fluoroquinolones like ciprofloxacin attain sufficient concentrations in milk and urine after systemic administration, thus making them more suitable for the treatment of mastitis, urinary tract infections and other systemic infections in cows (Jayakumar, 1998).

Clinically, fluoroquinolones are well tolerated, but CNS disorders including confusion, hallucination, anxiety, nervousness and seizures have been reported in two per cent of population. (Anastasio, 1988; Christ, 1990).

2.2 Norfloxacin

Norfloxacin is a second generation fluoroquinolone compound featuring a fluorine atom in position-6 and piperazinyl substituent at position-7 of the quinolone nucleus (Wentland, 1990).



Structure of Norfloxacin

2.2.1 Chemistry

Chemically, norfloxacin is 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid. The molecular formula is $C_{16}H_{18}FN_3O_3$ and the molecular weight is 319.3.

2.2.2 Physicochemical properties

Norfloxacin is white or pale yellow hygroscopic, photosensitive, crystalline powder and is readily soluble in aqueous solutions at pH 2-5 and also at pH 9 and above. At pH 7, it is sparingly soluble in aqueous solutions. It contains both acidic and basic groups and thus behaves as zwitterion. Norfloxacin, like all other fluoroquinolones is amphoteric in nature and has pK_a values at 6.3 and 8.8 (Dorflinger *et al.*, 1986).

2.2.3 Mechanism of action

Norfloxacin acts by inhibiting the enzyme DNA gyrase (Topoisomerase II), an enzyme responsible for controlling supercoiling in the bacteria (Wolfson and Hooper, 1985; Hawkey, 2003). DNA gyrase plays a vital role in DNA packing, replication and transcription (Martinez *et al.*, 2006). Mammals also possess an enzyme that is similar to DNA gyrase but it does not supercoil the DNA and is not affected by fluoroquinolones (Brown, 1996). Topoisomerase II is the major target for fluoroquinolones in Gram-negative bacteria, whereas topoisomerase IV

may be the primary target in Gram-positive bacteria (Hawkey, 2003). It has a broad spectrum of activity and is more potent *in vitro* than the non-fluorinated quinolone, nalidixic acid. However its activity may be reduced in acidic media.

2.2.4 Spectrum of activity

Similar to other fluoroquinolone compounds norfloxacin possess a wide spectrum of activity, excellent tissue penetration and is rapidly bactericidal at low concentrations often at a minimum inhibitory concentration (MIC) of less than 0.1 µg.ml⁻¹ (Fernandes, 1988).

Among Gram-negative aerobic bacteria norfloxacin is active *in vitro* against *Enterobacteriaceae* including *Escherichia coli* and *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Providencia* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp., and *Yersinia* spp. It is also active against *Pseudomonas aeruginosa*, but less so against other *Pseudomonas* species. *Haemophilus ducreyi*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Neisseria gonorrhoeae* are all very sensitive, including beta-lactamase producing strains. *Neisseria meningitidis* is also susceptible. Other Gram-negative aerobic bacteria reported to be sensitive to norfloxacin include *Pasteurella multocida*, *Helicobacter pylori*, *Acinetobacter* spp., *Campylobacter* spp., *Legionella* spp. and *Vibrio* spp.

Among Gram-positive aerobic bacteria, norfloxacin is active against *Staphylococci* including penicillinase-producing and penicillinase-non producing strains, and against some methicillin resistant strains, *Streptococci*, in particular *Streptococcus pneumoniae* and *Enterococci* spp. are less susceptible. Activity of norfloxacin against anaerobic bacteria is poor and its activity against Gram-negative bacteria is relatively more than against Gram-positive cocci (Goldstein, 1987).

The MIC₉₀ values of norfloxacin against various bacterial species and or isolates were reported by Sharma *et al.* (1994) and the recorded values of MIC₉₀ (mg/L) is presented in Table-1.

2.2.5 Post – antibiotic effect (PAE)

PAE is a well-established pharmacodynamic parameter that reflects an arrested bacterial growth following the removal of the active antibacterial agent from the growth medium. Fluoroquinolones including norfloxacin exert PAE in susceptible bacteria. The dosage interval can be extended for antibiotics exhibiting PAE effect resulting in less frequent administration of drugs and minimal toxicity to the host.

Renneberg and Walder (1989) recorded the PAE of norfloxacin (*in vitro*) against *Staphylococcus aureus* (2.9 ± 0.4 h), *Escherichia coli* (1.9 ± 0.2 h), *Streptococcus faecalis* (1.1 ± 0.3 h) and *Pseudomonas aeruginosa* (2.4 ± 0.3 h). Similarly, the *in vivo* PAE of norfloxacin (3 mg/kg, p.o) in Balb/c mice infected with above pathogens were respectively 4.3 ± 0.4 , 3.4 ± 0.3 , 3.3 ± 0.2 and 3.7 ± 0.2 h.

2.2.6 Pharmacokinetics of norfloxacin in general

Like other fluoroquinolones, absorption of norfloxacin in ruminants is erratic when compared to simple stomached animal species (Rylander and Norrby, 1983; Rao and Tripathi, 1999). The absolute bioavailability of orally administered norfloxacin in humans and in laboratory animals is 40 per cent. The presence of food can delay absorption. Bioavailability of norfloxacin is also affected by concomitant administration of conventionally used antacids and sucralfate. The relative bioavailability of norfloxacin was 1.8 and 56.6 per cent respectively in human volunteers given sucralfate concomitantly or two hours later (Parpia *et al.*, 1988). The plasma half life ($t_{1/2\beta}$) is three to four hours, which may be prolonged during renal impairment. The protein

Table-1: MIC₉₀ values of norfloxacin against various bacterial species and/ or isolates

Organism(s)	Norfloxacin, MIC ₉₀ (mg/L)
Enterobacteriaceae	
<i>Escherichia coli</i>	0.05 – 1; 0.12*
<i>Klebsiella pneumoniae</i>	0.2 – 0.5; 0.5*
<i>Salmonella typhi</i>	0.3
<i>Salmonella</i> spp.	0.3
<i>Shigella</i> spp.	0.03 – 0.3
<i>Proteus mirabilis</i>	0.25
<i>Proteus vulgaris</i>	0.06 – 0.25
Neisseriae	
<i>Neisseria gonorrhoea</i>	0.06
<i>Neisseria meningitidis</i>	0.1
Other Gram-negative bacterial species	
<i>Pseudomonas aeruginosa</i>	0.4 – 2.0; 2.0*
<i>Campylobacter jejuni</i>	0.2
<i>Vibrio parahaemolyticus</i>	0.05
<i>Haemophilus influenzae</i>	0.06 – 0.2; 0.06*
<i>Haemophilus ducreyi</i>	< 0.06
<i>Brucella</i> spp.	NA
Gram-positive bacteria	
<i>Staphylococcus aureus</i>	1-2
Coagulase negative <i>Staphylococci</i>	1
<i>Streptococcus</i> gp. A,B,C,G	4-8
<i>Streptococcus faecalis</i>	8
<i>Corynebacterium</i> spp.	NA
Anaerobic bacteria	
<i>Bacteroides fragilis</i>	32; >64*
<i>Fusobacterium</i> spp.	16
<i>Clostridium</i> spp.	64
Mycobacteria	
<i>Mycobacterium tuberculosis</i>	2
<i>Mycobacterium kansasii</i>	4
<i>Mycobacterium avium-intracellulare</i>	> 16
Others:	
<i>Mycoplasma pneumoniae</i>	6-12.5; 16*
<i>Chlamydia trachomatis</i>	16; 16*
<i>Ureaplasma urealyticum</i>	32; 16*

NOTE: NA = Not available; * Andersson and MacGowan, 2003

binding of norfloxacin was reported to be 14 per cent (Prescott and Baggot, 1993).

Norfloxacin is widely distributed in the body. It penetrates well into tissues of the genito-urinary tract and crosses the placenta as well. Relatively high concentrations are achieved in bile.

Norfloxacin undergoes both Phase-I metabolism (oxidation) as well as Phase II metabolism (glucuronidation and acetylation). In most species the principle metabolite(s) includes oxo-norfloxacin, norfloxacin glucuronide and N-acetylnorfloxacin (Anadon *et al.*, 1995; Rao and Tripathi, 1999).

Most of the absorbed dose of norfloxacin was excreted unchanged in urine and excreted by tubular secretion and glomerular filtration (Holmes *et al.*, 1985). In some species biliary excretion was found to be a significant route of elimination (Gilfillan *et al.*, 1984).

2.2.6.1 Equines (Horse and Donkey)

Pharmacokinetics of norfloxacin-glycine acetate was studied in horse after single intramuscular (i.m) administration at a dose rate 5 mg/kg body weight (Park *et al.*, 1997). The elimination half-live ($t_{1/2\beta}$), area under the curve (AUC), mean residence time (MRT), steady state volume of distribution (V_{dss}) were respectively 6.74 h, 4.36 mg.h.kg⁻¹, 5.23 h and 2.01 L.kg⁻¹. The peak concentration (C_{max}) of 0.57 µg.ml⁻¹ was obtained at 0.55 h (t_{max}) and the bioavailability was 53 per cent following i.m. administration.

Lavy *et al.* (1995) studied the disposition kinetics and comparative bioavailability of norfloxacin nicotinate after i.v, i.m and p.o administration in donkeys. After single i.v administration (10 mg/kg), the $t_{1/2\beta}$ was found to be 209±36 min. The apparent volume of distribution

($V_{d(\text{area})}$) and total body clearance (Cl_B) rate were respectively $3.34 \pm 0.58 \text{ L.kg}^{-1}$ and $1.092 \pm 0.123 \times 10^{-2} \text{ ml.min}^{-1}.\text{kg}^{-1}$. About 40 per cent of i.v. dose was recovered in urine as parent drug.

The bioavailability after p.o administration of norfloxacin nicotinate at a dose rate of either 10 or 20 mg/kg was observed to be poor (respectively 9.6 and 6.4%), while it was increased to 31.5 and 18.8 per cent respectively following i.m. administration.

2.2.6.2 Dogs

Brown *et al.* (1990) studied the pharmacokinetic profile of norfloxacin in dogs after single i.v. and single or multiple p.o administration. Following single i.v. administration of norfloxacin @ 5 mg/kg, the $t_{1/2\beta}$, $t_{1/2\alpha}$, AUC, MRT, V_{dss} were respectively 3.56 h, 0.467 h, $17.7 \pm 7.19 \mu\text{g.h.ml}^{-1}$, $4.21 \pm 1.10 \text{ h}$, $1.37 \pm 0.596 \text{ L.kg}^{-1}$. The AUC, MRT and bioavailability after p.o administration of similar dose were $4.77 \pm 5.26 \mu\text{g.h.ml}^{-1}$, $6.38 \pm 3.10 \text{ h}$ and 35 per cent respectively.

No difference in any pharmacokinetic variable was observed between single and multiple dose (5 mg/kg every 12 h for 14 days, p.o) of norfloxacin in dogs. The values of AUC and MRT following single ($5.38 \pm 1.58 \mu\text{g.h.ml}^{-1}$ and $5.56 \pm 1.78 \text{ h}$) or multiple dose ($4.61 \pm 4.41 \mu\text{g.h.ml}^{-1}$ and $5.07 \pm 1.53 \text{ h}$) respectively did not differ significantly ($P < 0.05$).

2.2.6.3 Pigs

The pharmacokinetics of norfloxacin glycine acetate was investigated (Chang *et al.*, 2007) in pigs after a single i.v or p.o administration @ 7.2 mg/kg body weight. Following i.v. dose, the $t_{1/2\beta}$, $t_{1/2\alpha}$, AUC, V_{dss} were respectively $7.42 \pm 3.55 \text{ h}$, $0.36 \pm 0.07 \text{ h}$, $9.66 \pm 2.55 \mu\text{g.h.ml}^{-1}$ and $4.66 \pm 1.39 \text{ L.kg}^{-1}$.

After single p.o administration the $C_{\max}$, $t_{\max}$, $t_{1/2\alpha}$, $t_{1/2\beta}$, AUC were $0.43 \pm 0.06 \mu\text{g} \cdot \text{ml}^{-1}$, $1.36 \pm 0.39 \text{ h}$, $0.78 \pm 0.27 \text{ h}$, $7.13 \pm 1.41 \text{ h}$ and $3.47 \pm 1.11 \mu\text{g} \cdot \text{h} \cdot \text{ml}^{-1}$ respectively with a mean systemic bioavailability of 31.10 per cent.

Shem Tov and Ziv (1994) studied the pharmacokinetic characterization of norfloxacin nicotinate in swine following systemic administration. Following i.v. administration, the $t_{1/2\beta}$, $t_{1/2\alpha}$, MRT and V_{dss} were 2.1 h, 4.2 min, $2.9 \pm 0.6 \text{ h}$ and $3.2 \pm 0.11 \text{ L} \cdot \text{kg}^{-1}$. While the $t_{1/2\beta}$ values after either i.m or s.c. administration were 4.45 h. Bioavailability was 51-64 per cent. Less than 20 per cent and 25 per cent of the dose were excreted in the urine as parent drug during the first 24 h after i.v. and s.c. administration respectively.

Anadon *et al.* (1995) studied the tissue residue of norfloxacin and its N-desethyl and oxo-metabolites in healthy pigs. After i.m and i.v. administration of norfloxacin @ 8 mg/kg, the plasma concentration-time profile was best described by two-compartment open model. After single i.m. administration, norfloxacin was absorbed rapidly with a $t_{\max}$ of $1.46 \pm 0.06 \text{ h}$. The $t_{1/2\beta}$ and MRT of norfloxacin in plasma were $4.99 \pm 0.28 \text{ h}$ and $6.05 \pm 0.22 \text{ h}$, respectively after i.m. administration and $3.65 \pm 0.16 \text{ h}$ and $3.34 \pm 0.16 \text{ h}$, respectively after i.v. administration.

2.2.6.4 Cattle

Gips and Soback (1996) studied the disposition kinetics of norfloxacin nicotinate in unweaned and weaned calves after single i.v. administration @ 7.5 mg/kg body weight. The body clearance rate was significantly ($P < 0.05$) higher in the weaned calves ($11.7 \pm 3.2 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) than in the unweaned calves ($8.5 \pm 2.0 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$), while the MRT was significantly smaller in the weaned calves ($185 \pm 79 \text{ min}$) when compared to unweaned calves ($211 \pm 33 \text{ min}$). The $V_{\text{d(area)}}$ was 1.8 ± 0.3 and

2.0±0.7 L.kg⁻¹ in unweaned and weaned calves, respectively. No significant variation in all other pharmacokinetic parameters were observed between two groups.

Gips and Soback (1999) established single dose pharmacokinetic variables for norfloxacin nicotinate after i.v. administration @ 10 mg/kg body weight in cows with healthy udder and in cows with chronic, subclinical or post-acute clinical mastitis. The author has observed significant ($P<0.01$) variations in pharmacokinetic parameters between cows with healthy and infected udder quarters. The $t_{1/2\beta}$, V_{dss} and MRT were 353 min, 3.1±0.7 L.kg⁻¹ and 306±76 min in control group, 206 min, 2.2±0.6 L.kg⁻¹ and 168±39 min in cows with sub clinical mastitis and 115 min, 1.3±0.2 L.kg⁻¹ and 95±9.0 min in cows with post-acute clinical mastitis.

The clearance of norfloxacin was significantly ($P<0.01$) more in cows with diseased udder as compared to healthy udder quarters. The body clearance (Cl_B) rates in healthy, sub-clinical and clinical mastitis cows were respectively 10.4±2.5, 13.2 ±1.9 and 14.2±2.1 ml.min⁻¹.kg⁻¹.

The concentration of norfloxacin in milk was relatively high as compared to corresponding serum values. The AUC (milk) of norfloxacin was significantly ($P<0.01$) less in infected udder quarters of cows belonging to mastitis group (5075±1887 mg.min.L⁻¹ and 7484±4645 mg.min.L⁻¹ in the sub-clinical and clinical mastitis respectively) when compared to cows with healthy udder quarters (23899±6206 mg.min.L⁻¹).

2.2.6.5 Sheep and goats

Soback *et al.* (1994) studied the pharmacokinetic profile of norfloxacin in ewes following single i.v. administration @ 8 mg/kg. The

$t_{1/2\beta}$, AUC, Cl_B were respectively 266 min, 7119 $\mu\text{g.h.ml}^{-1}$ and 4.2 ml.min.kg^{-1} .

Prakash (2007) described the pharmacokinetic profile of norfloxacin in adult sheep after i.v. administration @ 7.5 mg/kg body weight. The $t_{1/2\beta}$, AUC, MRT, $V_{d(\text{area})}$, V_{dss} , and Cl_B were 1.76 ± 0.04 h, 14.54 ± 0.66 $\mu\text{g.h.ml}^{-1}$, 2.30 ± 0.09 h, 1.31 ± 0.08 L.kg^{-1} , 1.19 ± 0.10 L.kg^{-1} and 517.0 ± 24.04 $\text{ml.kg}^{-1}.\text{h}^{-1}$ respectively. The ratio of tissue concentration to that of plasma concentration (T/P) was found to be 0.57.

Jha *et al.* (1994) studied the kinetic profile of norfloxacin in afebrile and febrile goats after a single i.v. dose @ 5 mg/kg. Mean peak plasma concentrations of 62.25 ± 1.23 and 75.46 ± 0.72 $\mu\text{g.ml}^{-1}$ respectively was attained at 2 min in afebrile and febrile goats. The values of $t_{1/2\beta}$ and $t_{1/2\alpha}$ were 0.25 ± 0.02 h, 0.07 ± 0.01 h in afebrile and 0.24 ± 0.01 h, 0.16 ± 0.01 h in febrile goats respectively. The AUC was significantly ($P < 0.05$) higher in febrile goats (24.49 ± 0.75 $\mu\text{g.h.ml}^{-1}$) when compared to afebrile goats (18.97 ± 1.06 $\mu\text{g.h.ml}^{-1}$). The body clearance rate was higher in afebrile goats (4.45 ± 0.23 $\text{ml.min}^{-1}.\text{kg}^{-1}$) as compared to febrile ones (3.15 ± 0.09 $\text{ml.min}^{-1}.\text{kg}^{-1}$). However, the MRT was similar (0.32 ± 0.01 h) in both groups.

Wajecha and Javed (2006) determined the bioavailability and pharmacokinetics of two commercially available formulations of norfloxacin in goats after single i.m. administration @ 5 mg/kg body weight. The mean value of AUC of formulation-A (7.57 ± 1.10 $\mu\text{g.h.ml}^{-1}$) was significantly ($P < 0.01$) higher than for formulation-B (5.05 ± 1.50 $\mu\text{g.h.ml}^{-1}$). The bioavailability was also significantly ($P < 0.05$) higher for formulation-A ($169.0 \pm 2.40\%$) when compared to formulation-B ($66.90 \pm 2.04\%$). However, the other pharmacokinetic parameters non-significantly differed between formulations.

2.2.6.6 Chicken

Pharmacokinetics of norfloxacin in broiler chickens after single p.o or i.v. administration @ 8 mg/kg body weight was investigated by Anadon *et al.* (1992). The pharmacokinetic variables $t_{1/2\beta}$, V_{dss} , AUC, MRT and Cl_B after p.o and i.v. administration were 12.08 ± 0.59 h v/s 8.0 ± 0.3 h; 9.43 ± 0.17 v/s 3.14 ± 0.06 L.kg⁻¹; 12.66 ± 0.54 v/s 22.14 ± 0.4 mg.h⁻¹.L⁻¹; 15.01 ± 0.81 v/s 8.71 ± 0.23 h and 0.64 ± 0.03 v/s 0.36 ± 0.01 L.h⁻¹.kg⁻¹ respectively. The C_{max} , t_{max} and bioavailability following oral administration of norfloxacin were respectively 2.89 ± 0.20 µg.ml⁻¹, 0.22 ± 0.02 h and 57 per cent.

The metabolism of norfloxacin after oral administration was also investigated by the author. The major metabolites: N desethylene norfloxacin and oxo-norfloxacin concentration in plasma reached a peak somewhat later (0.86 h and 1.06 h respectively) than did that of norfloxacin (0.22 h). However, the plasma elimination half-lives of both the metabolites were similar (10.0 h v/s 9.95 h), but less than that observed for norfloxacin (12.08 h).

Laczay *et al.* (1998) have reported comparative pharmacokinetics of norfloxacin in chickens, turkeys and geese after a single p.o administration @ 10 mg/kg body weight using a commercial formulation (20% oral solution) containing the norfloxacin base.

In chicken, the kinetic variables, *viz*: C_{max} , t_{max} , AUC, MRT were respectively 1.46 ± 0.18 µg.ml⁻¹, 1.99 ± 0.17 h, 10.41 ± 1.22 µg.h.ml⁻¹ and 8.82 ± 1.02 h. The disappearance of norfloxacin from plasma was slow (11.1 h) following single oral dose.

In turkeys, the measured plasma concentrations were considerably lower than in chickens. The C_{max} was 0.95 ± 0.15 µg.ml⁻¹ reached at 1.94 ± 0.08 h. The $t_{1/2\beta}$, AUC, MRT were 9.07 ± 1.0 h, 5.97 ± 0.98 µg.h.ml⁻¹, 6.64 ± 1.08 h respectively.

In geese, mean $C_{\max}$ was $1.58 \pm 0.30 \mu\text{g} \cdot \text{ml}^{-1}$ observed at 2.39 ± 0.18 h. The $t_{1/2\beta}$, AUC, MRT were 10.65 ± 2.41 h, $14.78 \pm 3.03 \mu\text{g} \cdot \text{h} \cdot \text{ml}^{-1}$, 9.96 ± 1.16 h respectively.

2.2.6.7 Rabbits

Park *et al.* (1997) studied the pharmacokinetics of norfloxacin glycine acetate in New Zealand white rabbits after oral administration @ 5 mg/kg body weight. The $t_{1/2\beta}$, AUC, V_{dss} MRT were respectively 3.93 h, $3.20 \text{ mg} \cdot \text{h} \cdot \text{kg}^{-1}$, $3.77 \text{ L} \cdot \text{kg}^{-1}$, 3.11 h. The $C_{\max}$, $t_{\max}$ and bioavailability following p.o administration were $0.82 \mu\text{g} \cdot \text{ml}^{-1}$, 0.58 h and 26 per cent respectively.

Rylander and Norrby (1983) studied serum disposition profile of norfloxacin after oral administration @ 100 mg/kg body weight in rabbits using tissue-cage model. The serum concentration of 1.71 ± 0.37 , 2.01 ± 0.31 , 1.76 ± 0.32 , 1.04 ± 0.35 , 0.69 ± 0.30 and $0.53 \pm 0.28 \mu\text{g} \cdot \text{ml}^{-1}$ was observed at 0.5, 1, 2, 4, 6 and 8 h after single oral dose. The tissue concentrations of norfloxacin were 0.24 ± 0.04 , 0.37 ± 0.08 , 0.55 ± 0.07 , 0.56 ± 0.10 and $0.54 \pm 0.11 \mu\text{g} \cdot \text{ml}^{-1}$ at 1, 2, 4, 6 and 8 h respectively.

2.2.6.8 Rats

Park *et al.* (1997) studied the pharmacokinetics of norfloxacin-glycine acetate in rats after p.o @ 5 mg/kg body weight. The $C_{\max}$, $t_{\max}$, $t_{1/2\beta}$, AUC, V_{dss} were $0.79 \mu\text{g} \cdot \text{ml}^{-1}$, 0.19 h, 2.76 h, $3.25 \text{ mg} \cdot \text{h} \cdot \text{kg}^{-1}$, $6.23 \text{ L} \cdot \text{kg}^{-1}$ respectively.

2.3 Bioenhancers in therapeutics

A 'bioenhancer' may be defined as a substance that can increase the efficacy of chemotherapeutics (anticancer, antibiotics, antiviral drugs etc.) to a clinically appreciable amount. The action of bioenhancers was first documented by Bosc (1929) who described that

addition of long pepper (*Piper longum*) to Vasaka (*Adhatoda vasica*) increased the anti-asthmatic property of *Adhatoda vasica* leaves.

2.3.1 Piperine

Piperine is an alkaloid found naturally in plants belonging to *Piperaceae* family. Piperine is generally isolated from the fruit of black pepper (*Piper nigrum* L.) or long pepper (*Piper longum* L.) or rhizomes of ginger (*Zingiber officinalis*).

There is evidence that piperine increases bioavailability of certain drug molecules by virtue of its non-specific inhibition of enzymes concerned with drug metabolism (Annamalai and Manavalan, 1989). Reen *et al.* (1996) suggested that piperine mediated inhibition of benzo(a) pyrene metabolism and inducible arylhydrocarbonhydroxylase activity is due to its direct interaction with CYP1A1 in the rat hepatoma cell line. Additionally, the bioenhancer property of piperine has been attributed to its influence on CYP3A4 and P-gp protein (Bhardwaj *et al.*, 2002). The treatment of *Piper longum* or its active principle piperine was reported to increase the rate and extent of absorption of some of the therapeutic agents which are moderately absorbed on their own, by enhancement of blood supply through vasodilation (Atal *et al.*, 1980; Annamalai and Manavalan 1989).

Co-administration of piperine has been reported to enhance the blood level of oxyphenylbutazone in rats (Majumbar *et al.*, 1999) and nimesulide in rats (Gupta *et al.*, 2000). Similarly, Zutshi *et al.* (1985) observed increased blood levels of rifampicin in patients when it was administered with piperine.

Administration of piperine can influence the pharmacokinetic variables of antimicrobial agents belonging to different class. Hiwale *et al.* (2002) observed increased bioavailability of amoxycillin trihydrate and

cefatoxime sodium in rats following concomitant administration of piperine. Ramesh *et al.* (2001) reported increased persistence and bioavailability of enrofloxacin and its metabolite ciprofloxacin upon co-administration with piperine in goats.

Singh *et al.* (2005) studied pharmacokinetic profile of oxytetracycline (10 mg/kg body weight) following seven days post-oral treatment of *Piper longum* in White Leghorn hens. Prior treatment of *Piper longum* significantly ($P < 0.01$) reduced the elimination rate constant (β) and increased elimination half-life ($t_{1/2\beta}$). Thus the body clearance (Cl_B) reduced by 21 per cent whereas total duration of pharmacological effect (t_d) increased by 29 per cent. Thus piperine treatment has reduced the loading and maintenance dose by 33.3 and 39 per cent respectively.

Trikatu®, a commercial preparation (Indian Herbs, UP, India) containing mixture of *Piper longum*, *Piper nigrum* and *Zingiber officinalis* was demonstrated to enhance the per-oral bioavailability of pefloxacin in goats (Madhukar, 2006).

2.3.2 Curcumin

Curcumin, a yellow pigment is a major bioactive component of turmeric plant (*Curcuma longa* L.). Turmeric is a medicinal plant extensively used in *Ayurveda*, *Unani* and *Siddha* medicine as home remedy for various diseases. Turmeric is a major food additive (spice), preservative and colouring agent in Asian countries, including China and South East Asia. It is called by different vernacular names namely, 'Turmeric' in English, 'Haldi' in Hindi, 'Arishina' in Kannada, 'Jiang huang' in Chinese 'Gelbwurz' or 'Kurkuma' in German, 'Kurkum' in Arabic language.

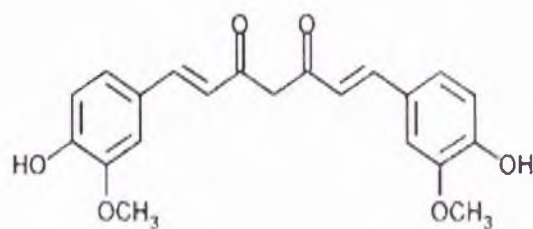
Turmeric as powder is composed of protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). The

essential oil (5.8%) obtained by steam distillation of rhizome of turmeric plant contains 3 to 4 per cent curcuminoids *viz*: curcumin (94%), demethoxycurcumin (6%) and bis-demethoxycurcumin (0.3%) (Chattopadhyay *et al.*, 2004). Among curcuminoids, curcumin possess major therapeutic properties.

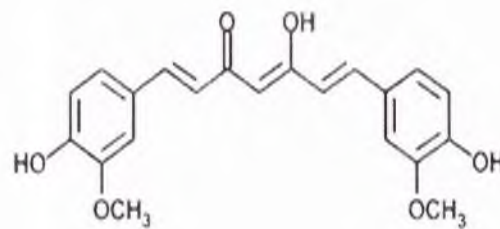
2.3.2.1 Structure and chemistry

Curcumin was first isolated by Vogel and Pelletier (1815) and its chemical structure was determined by Roughley and Whiting in 1973.

The curcuminoids are polyphenols and are responsible for yellow colour of turmeric. The active principle curcumin is a flavonoid. Curcumin can exist in at least two tautomeric forms, *-keto* and *-enol*.



Curcumin *-enol* form



Curcumin *-keto* form

Structure of Curcumin

The *-enol* form is more energetically stable in the solid phase and in solution. Curcumin incorporates several functional groups. The aromatic ring systems, which are polyphenols are connected by two α , β – unsaturated carboxyl groups and the two carboxyl groups form a diketone.

Chemical name : 1,7-bis (4-hydroxy-3-methoxyphenyl) hepta-1, 6-dien-3, 5-dione

Molecular formula : $C_{21}H_{20}O_6$

Molecular weight : 368.4

Curcumin is sparingly soluble in water, where as its semisynthetic analogues like curcumin sodium phenate are water soluble. Curcumin is a highly fragile molecule, photo-degradable and susceptible to extreme alkaline and acidic conditions.

2.3.2.2 Safety and toxicity studies

The average intake of turmeric by Asians varies from 0.5 to 1.5 g/day/person, which produces no toxic symptoms (Eigner and Scholz, 1999). Male and female Wistar rats, guinea pigs and monkeys fed with turmeric at much higher doses (2.5 g/kg body weight) did not show behavioural as well as any pathological changes in kidney, liver and heart (Holder *et al.*, 1978). Oral ingestion of turmeric powder at the rate of 8 g/day had no adverse effects in man according to World Health Organisation.

Similarly, curcumin given to Wistar rats, guinea pigs and monkeys of both sexes at a dose of 300 mg/kg body weight (p.o) did not reveal pathological, behavioural abnormalities or any lethality (Bhavani Shankar *et al.*, 1980). Human clinical trials also indicate that curcumin has no toxicity when administered at doses of 1-8 g/day and 10 g/day (Aggarwal *et al.*, 2003). Oral LD₅₀ of curcumin in mice was found to be more than 2 g/kg body weight (Kohli *et al.*, 2005).

2.3.2.3 Pharmacological properties

Curcumin is known to possess a wide range of pharmacological properties and few of them are routinely exploited in therapeutics. Pharmacodynamic studies revealed anti-inflammatory (Arora *et al.*, 1971); antibacterial (Negi *et al.*, 1999), antiviral (Bourne *et al.*, 1999), antifungal (Apisariyakul *et al.*, 1995); antispasmodic (Itthipanichpong *et al.*, 2003); hepatoprotective (Park *et al.*, 2000); antioxidant (Sharma, 1976; Joe and Lokesh, 1994); antitumour (Dceeb *et al.*, 2003; Chen *et al.*,

1996; Kawamori *et al.*, 1999) and anticarcinogenic (Chen and Huang, 1998) properties of curcumin in various experimental models.

Curcumin was reported to ameliorate aflatoxin-B₁ induced hepatotoxicity in mice (Prakash *et al.*, 2007). A study conducted by Kutluay *et al.* (2008) has showed that low concentrations of curcumin interferes with replication of herpes simplex virus-1. Further, curcumin also inhibited the recruitment of RNA polymerase II to viral DNA, thus inhibiting the transcription of the viral DNA.

2.3.2.4 Curcumin as bioenhancer

More recently, curcumin was reported to inhibit catalase and hepatic and gastrointestinal tract UDP glucuronyl transferase isoenzymes and thereby increase bioavailability of mylophenolic acid, an immunosuppressive agent (Owens *et al.*, 2004). Curcumin was also reported to induce changes in P *glycoprotein* (P-*gp*), a drug transporter protein and CYP 3A expression and hence curcumin exposure resulted in increased C_{max} and AUC of celiprolol (a P *gp* substrate with negligible CYP_{P450} metabolism) at 30 mg/kg (p.o). Similarly, curcumin administration (p.o) induced changes in P *gp* activity and CYP 3A expression and decreased the oral clearance of midazolam in rats (Wenxia *et al.*, 2006).

Further, curcuminoids are reported to induce glutathione S-transferase and are potent inhibitors of cytochrome P₄₅₀ enzymes (Wikimedia, 2008). Valentine *et al.* (2006) reported that curcumin had no effect on catalytic activities of hepatic catechol-o-methyltransferase, UDP glucuronyl transferase and ovarian aromatase following exposure to curcumin (200/400 mg/kg body weight, p.o) for one to two weeks. However, curcumin treatment (400 mg/kg) caused a 20 per cent increase in glutathione S-transferase activity.

The bioavailability of curcumin after 2 to 10 g of oral administration resulted in undetectable to very low serum levels (Shoba *et al.*, 1998). Curcumin is unstable in the gut and the traces that pass through the gut rapidly degrades or is conjugated through glucuronidation. Co-supplementation with 20 mg of piperine increased the absorption of curcumin from the gut significantly (Shoba *et al.*, 1998).

Apart from these reports, extensive perusal of literature did not reveal bioenhancer properties or mechanism(s) of action of curcumin or curcuminoids. Therefore, the present investigation was carried out to examine the benefits of curcumin exposure on disposition profile of norfloxacin, a broad spectrum fluoroquinolone compound in rabbits.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

3.1 Experimental animals

The study was conducted in 8-10 months old New Zealand white rabbits procured from commercial source. Animals were quarantined for one week and later they were dosed with albendazole (5 mg/kg, p.o), a broad spectrum antihelmintic. The experimental animals were reared in individual cages and fed standard rabbit feed (NIN, Hyderabad) and had access to water *ad libitum*. The animals were acclimatized to experimental laboratory animal house conditions for a period of three weeks before carrying out the studies. The experiment was initiated in rabbits when they attained a mean body weight of 1.65 ± 0.22 kg.

Necessary approval from Institutional Animal Ethics Committee (IAEC) was obtained (No. Dean/VCB/PG R/1249, dated 16-11-2007) before conducting the experiment.

3.2 Experimental design

The rabbits (N=16) of either sex were divided into two groups of eight each. The experimental design was as follows:

Group-I (Control, n=8)	Rabbits were administered with norfloxacin at the rate of 100 mg/kg, per orally
Group-II (Treated, n=8)	Rabbits were administered with curcumin @ 60 mg/kg, per orally for three days at an interval of 24 h followed by norfloxacin @ 100 mg/kg, per orally three hours after last dose of curcumin.

3.3 Drugs and Chemicals

Norfloxacin standard was obtained by M/s Aravind Laboratory (P) Ltd., Hyderabad. Curcumin (minimum, 94 % curcuminoid content) was obtained from Sigma, St. Luis, USA. Norfloxacin and Curcumin were protected from light to avoid photo-degradability during storage. HPLC grade water, acetonitrile and methanol were obtained from E-Merck, Chennai. All other chemicals used in the study were of analytical grade. All the solvents and solutions were filtered through 0.2 μ nylon membrane filter (Hi-media, Mumbai) before using them during HPLC analysis of norfloxacin.

3.4 Preparation of norfloxacin solution

The required amount of drug was dissolved in 0.1N HCl to obtain a 3.33 per cent solution of norfloxacin (50 mg of norfloxacin in 1.5 ml 0.1N HCl). Norfloxacin was administered @ 100 mg/kg body weight (p.o).

3.5 Administration of curcumin

Curcumin is a fine solid substance sparingly soluble in water. The required amount of curcumin was dissolved in a mixture of distilled water and Tween-20 at 2:1 ratio restricting the total volume to 4-5 ml. Each time fresh solutions were made and was administered (p.o) to rabbits (group-II) @ of 60 mg/kg body weight for a period of three days at 24 h interval.

3.6 Administration of norfloxacin and blood sampling

The rabbits in group-I received a single oral dose of norfloxacin @ 100 mg/kg body weight by means of disposable syringe fitted with non-corrosive and non-irritant catheter, physiological saline (5ml) was immediately flushed through the syringe after the administration of

norfloxacin each time. Animals in group-II received similar amount of norfloxacin three hours after last dose of curcumin. Rabbits in control group also received appropriate quantity of vehicle prior to norfloxacin administration. Feed was withheld at least six-to-eight hours before and until four hours after drug administration. Blood samples (1-1.5ml) were drawn from the marginal ear veins into heparin coated tubes (Hi-media, Mumbai) immediately before (0') and 5, 10, 15, 30, 45 minutes and 1, 1.15, 1.30, 2, 3, 4, 5, 6, 8, 12 and 24 hours after the administration of norfloxacin under aseptic conditions. A cotton gauze wet with xylene was used to engorge ear veins whenever difficulty arised while collecting blood samples. Plasma samples were obtained by centrifugation (5000 rpm, 10 min) of blood samples and later they were stored at - 20°C until assayed.

3.7 Assay of norfloxacin in plasma

Plasma concentration of norfloxacin was determined using High Performance Liquid Chromatography (HPLC; Shimadzu, Japan). Quantitative determination of norfloxacin in plasma was done according to the method described in British Pharmacopoeia (2006).

3.7.1 HPLC conditions

The HPLC system consists of double pump (LC-20AT), rheodyne manual injector with 20 µl loop, dual wavelength UV detector (SPD-20A) and LC Solution® software for data analysis. A reverse phase C₁₈ column (250 x 4.5 mm, Particle size 5±0.3µm, Pore diameter 100±10 Å, Phenomenax®, USA) served as a stationary phase. The mobile phase consisted of 0.1% v/v orthophosphoric acid (pH adjusted to 2.0) and acetonitrile mixed at a ratio of 850:150 volumes. The flow rate of mobile phase was 1ml per minute. The detection wavelength was set at 275 nm and the samples were analyzed for eight minutes at room temperature.

Plate 1: Experimental rabbits maintained in laboratory animal house

Plate 2: High performance liquid chromatography (HPLC) fitted with Rheodyne manual injector (LC 20AT; Shimadzu, Japan)



Plate-1

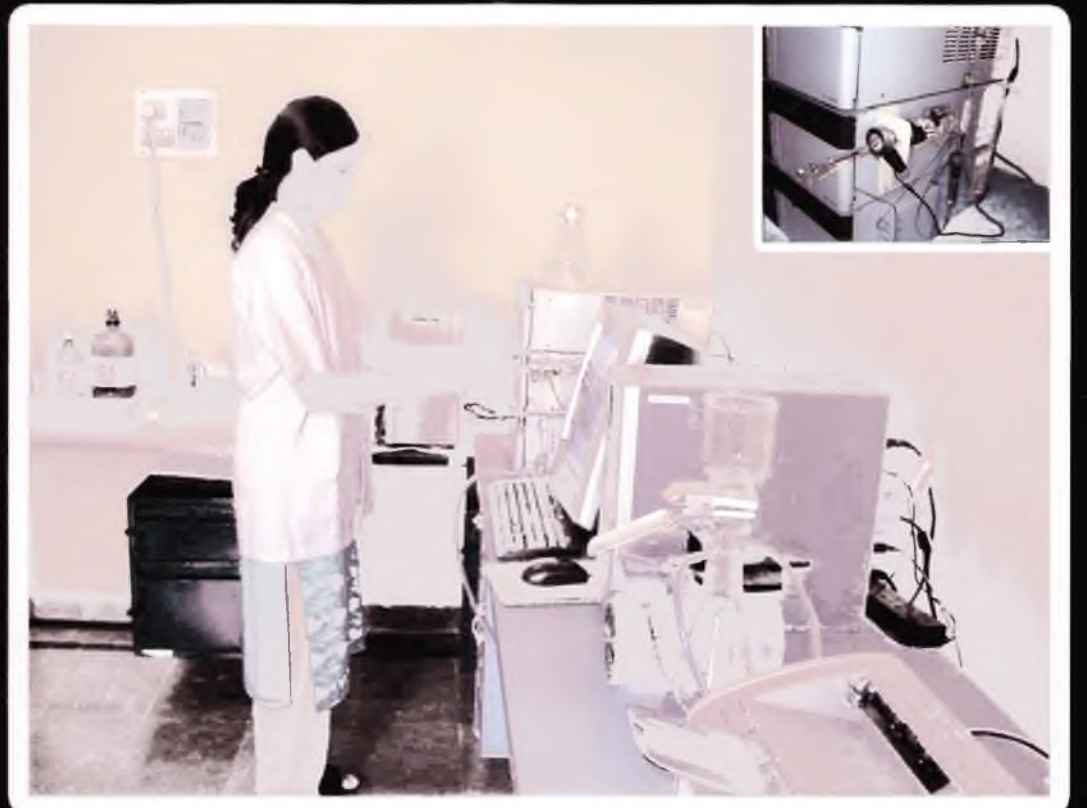


Plate-2

There were no interfering peaks in the chromatogram at the retention time ($R_t = 4.90 \pm 0.14$ min) of norfloxacin. Extracted plasma samples were injected manually to Rheodyne syringe system using Hamilton syringe.

3.7.2 Sample preparation

Norfloxacin was extracted from plasma samples by liquid liquid extraction technique. To 300 μ l of plasma, 450 μ l of acetonitrile was added (1:1.5) and mixed by vortexing for 30 seconds, the mixture was centrifuged at 5000 rpm for 12 minutes. The clear supernatant thus obtained was transferred into a 2 ml micro centrifuge tubes and later filtered through 0.2 μ HN nylon membrane filter. A 10 μ l of this was injected into HPLC system using Hamilton syringe. All plasma samples were analyzed in duplicate.

3.7.3 Construction of standard curve

Drug free plasma was obtained from rabbits prior to initiation of animal experiment for standardization and constructing standard curve of norfloxacin. The calibration curve was constructed in the range of 0.015 to 4 μ g/ml of norfloxacin in plasma. The standard curve was prepared by plotting peak area (x-axis) against concentration of drug (y-axis). The standard curve was linear in the range of 0.015 to 4 μ g/ml with R^2 value of 0.999. The limit of detection was 0.015 μ g/ml. A representative chromatogram of norfloxacin is depicted in Fig.1. The overlay report of various concentrations of norfloxacin and standard curve of norfloxacin are depicted in Fig.2 and Fig. 3 respectively.

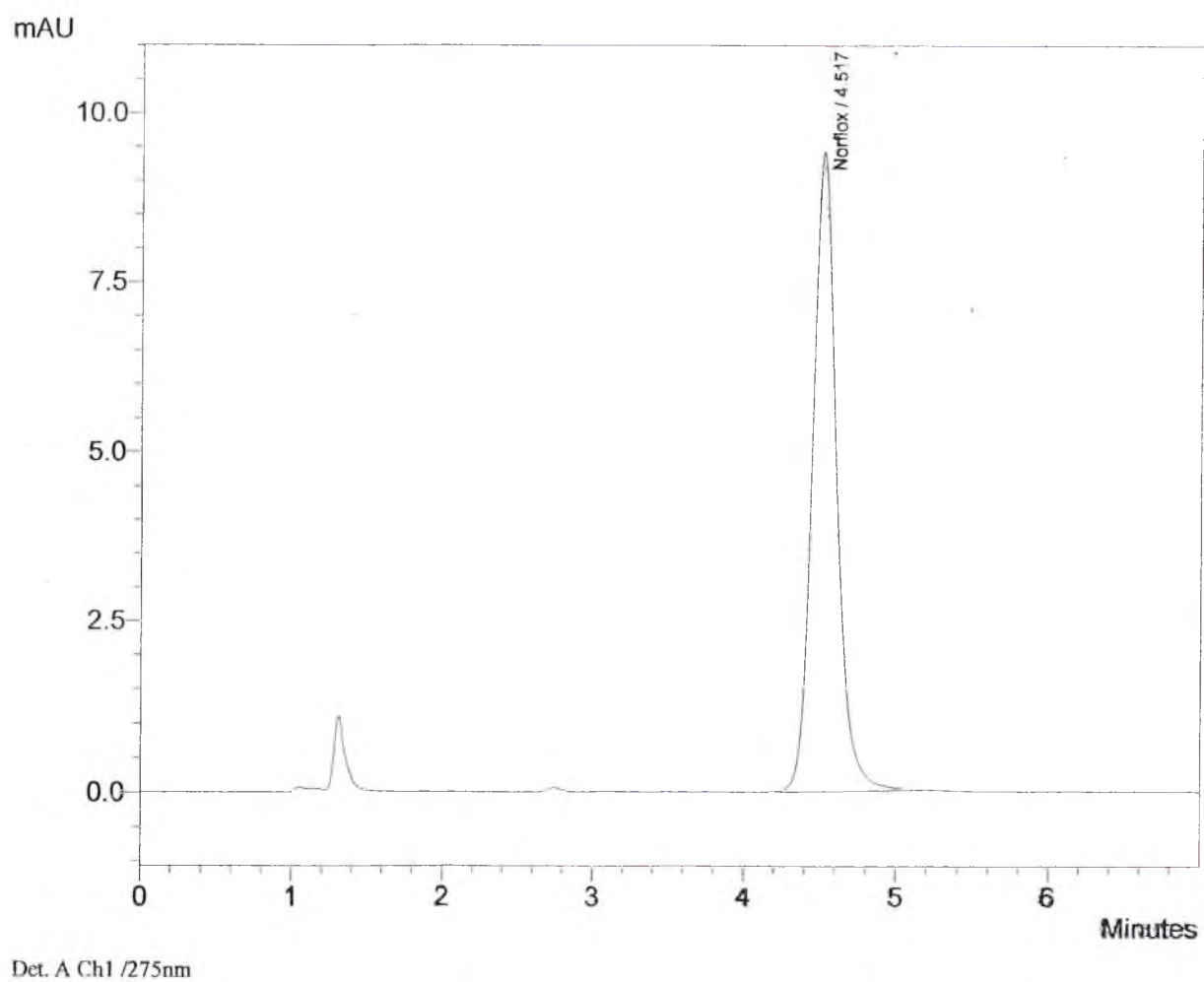
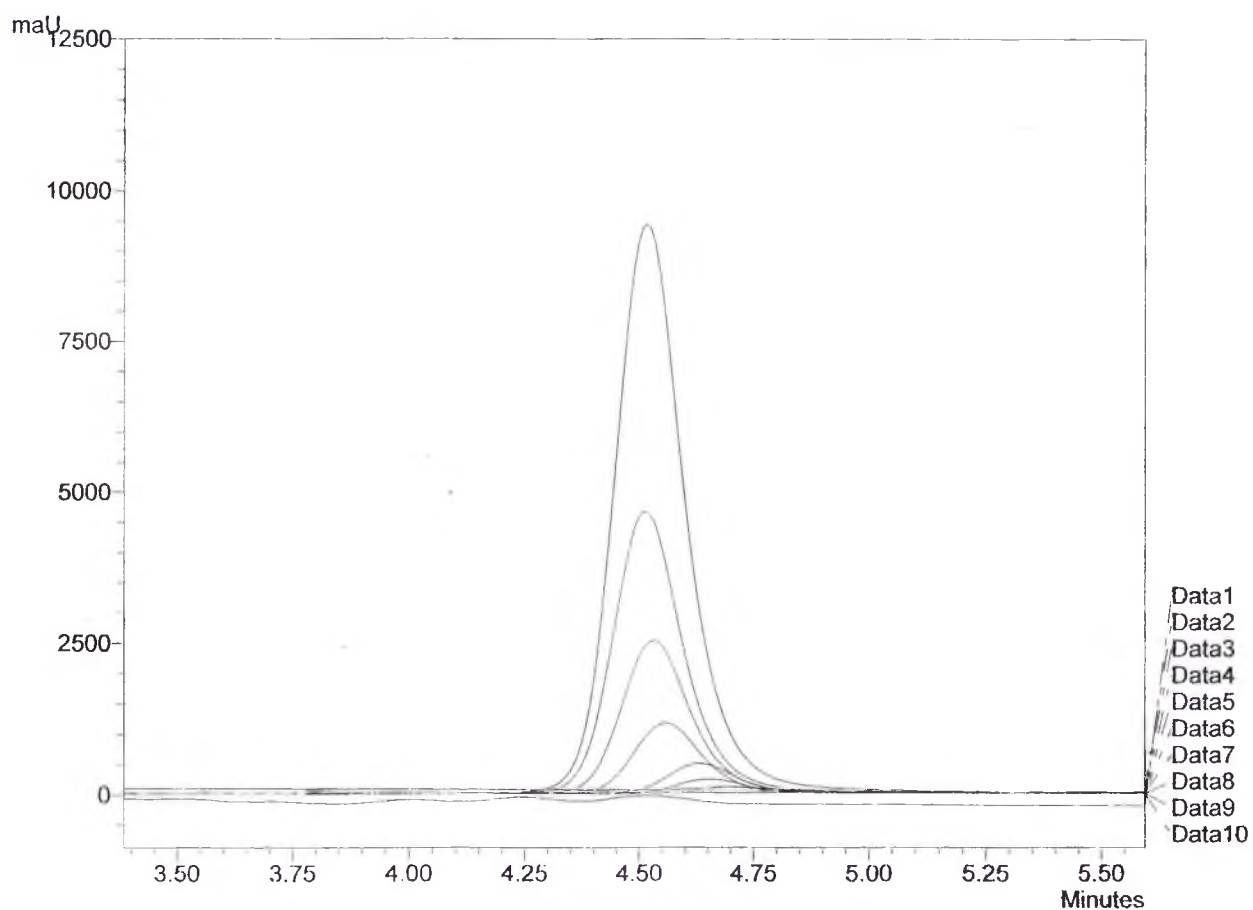


Fig.1: A representative chromatogram of norfloxacin



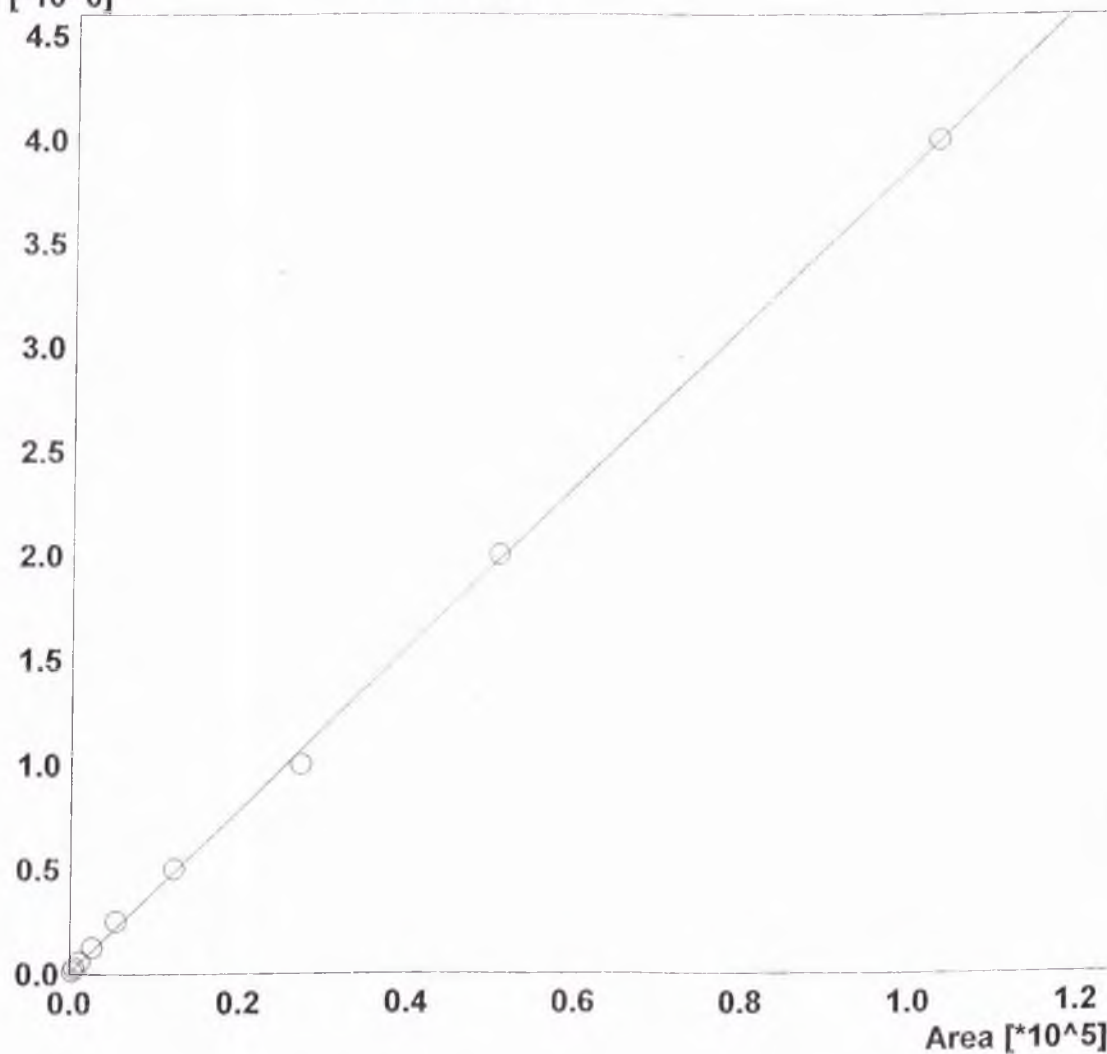
Note: Read from bottom to top

Data 1: Plasma blank
Data 2: Plasma standard 0.015 ug/ml
Data 3: Plasma standard 0.03 ug/ml
Data 4: Plasma standard 0.06 ug/ml
Data 5: Plasma standard 0.125 ug/ml
Data 6: Plasma standard 0.25 ug/ml
Data 7: Plasma standard 0.5 ug/ml
Data 8: Plasma standard 1.0 ug/ml
Data 9: Plasma standard 2.0 ug/ml
Data 10: Plasma standard 4.0 ug/ml

Fig.2: Overlay report of norfloxacin standard

Fig. 3 : Standard curve of norfloxacin

ID# : 1
Name : Norflox
Quantitative Method : External Standard
Function : $f(x)=3.87817e-005*x+0.0107785$
Rr1=0.9997762 Rr2=0.9995525
MeanRF:4.44812e-005 RFSD:5.87102e-006 RFRSD:1
FitType : Linear
ZeroThrough : Not Through
WeightedRegression : None
Detector Name : Detector A



3.7.4 Quantification

The linear regression formula obtained from calibration curve was used to derive the concentrations of norfloxacin in plasma by substituting respective peak area of the norfloxacin chromatogram.

$$X = a' + b' Y$$

Where, X = dependent variable (concentration in µg/ml)

a' = intercept

b' = regression coefficient X upon Y

Y = independent variable (peak area)

3.7.5 Analytical recovery

Analytical recovery for the method adopted was studied by using external standard technique. It was determined by adding norfloxacin to drug -- free rabbit plasma and to a solution of mobile phase to yield concentrations of 0.125, 0.5 and 2 µg/ml. Plasma samples were subjected to liquid -- liquid extraction. Both plasma and mobile phase standards were analyzed as described above. Recovery was calculated as ratio of peak areas obtained for plasma and those obtained for mobile phase based standards. For each concentration two determinants were made. Per cent recovery was calculated using following regression formula:

$$\% \text{ recovery} = \frac{n \sum XY - (\sum X) (\sum Y)}{n \sum X^2 - (\sum X)^2}$$

Where, X = concentration of drug spiked

Y = concentration found by assay method
(recovery x concentration spiked)

n = number of observations

Table-2 : Per cent recovery of norfloxacin in rabbit plasma

	Norfloxacin in plasma		
Concentration spiked (µg/ml)	0.125	0.5	2.0
Recovery (range)	0.07-0.12	0.23-0.49	0.96-1.30
Concentration found (µg/ml)	0.009-0.015	0.115-0.245	1.92-2.64
Mean ± SE (µg/ml)	0.012±0.004	0.18±0.09	2.28±0.51
Per cent recovery	94.17 %		

3.7.6 Assay precision accuracy

The precision of the analytical method adopted was determined by evaluating intra-day and inter-day variation. Intra-day variation was determined by assaying (in-triplicate) of three known concentrations (0.125, 1 and 4 µg/ml). Inter-day variation was determined by assaying three plasma standards (0.125, 1 and 4 µg/ml) on three different occasions at least 24 hours apart between each assay. The precision of the method was expressed as per cent co-efficient of variation (CV). The summary of the results obtained is presented in Table-3.

Table-3: Intra-day and inter-day assay co-efficient of variation (CV, %) of the HPLC method for norfloxacin

Concentration in plasma (µg/ml)	Intra-day assay CV(%)	Inter-day assay CV(%)
0.125	2.13	8.08
1.0	0.42	3.4
4.0	0.46	3.09

3.8 Pharmacokinetic analysis

The plasma concentration-time profile of norfloxacin of each experimental animal was used to determine its pharmacokinetics. The pharmacokinetic data of norfloxacin was analysed using 'method of least square' and 'method of residual yields' as described by Gibaldi and Perrier (1982). The compartmental analysis of the data was undertaken using the mono-exponential equation:

$$C_p^t = Be^{-\beta t} - Ae^{-K_a t}$$

Where, C_p^t = plasma drug concentration at time 't'

B = zero-time intercept of elimination phase

A = zero-time intercept of regression line of absorption phase

K_a = absorption rate constant

β = overall elimination rate constant

t = time

e = natural logarithm base

The absorption and elimination half-lives were derived from the equation: $0.693/k_a$ and $0.693/\beta$ respectively where in K_a and β are the rate constants for absorption and elimination phase.

The total area under the plasma concentration-time curve (AUC) and area under the first moment of plasma drug concentration-time curve (AUMC) were calculated according to Ritschel (1976).

$$AUC = \frac{B}{\beta} - \frac{A}{K_a}$$

$$AUMC = \frac{B}{\beta^2} - \frac{A}{K_a^2}$$

The mean residence time (MRT) was calculated by obtaining the ratio of AUMC : AUC

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}}$$

The total duration of pharmacological effect (t_d) was calculated according to Levy and Nelson (1965).

$$t_d = \frac{2.3}{\beta} \log \frac{A_o}{C_{p(\min)}}$$

Where, β = the overall elimination rate constant

A_o = dose (mg/kg)

$C_{p(\min)}$ = minimum effective concentration of drug

The bioavailability (F) of the drug after per-oral administration was calculated according to Notari (1987). The AUC and rate constant of elimination (β) recorded in the previous study (Prakash, 2007) following i.v. administration of norfloxacin in rabbits was substituted.

$$F = \frac{\text{AUC}_{(\text{oral})} \times \beta}{\text{AUC}_{(\text{i.v.})} \times \beta} \times 100$$

The volume of distribution $V_{d(\text{area})}$ and clearance from the body (Cl_B) were calculated as per the equation described (Gibaldi and Perrier, 1982) for non-vascular route of administration.

$$V_{d(\text{area})} = \frac{\text{Dose}}{\beta \cdot \text{AUC}} \times F$$

$$Cl_B = \frac{\text{Dose}}{\text{AUC}} \times F$$

The loading and maintenance dosage schedule so as to maintain a minimum inhibitory concentration (MIC) of 0.1, 0.5 and 1.0 $\mu\text{g.ml}^{-1}$ in plasma was calculated according to Notari (1987).

3.9 Integration of Pharmacokinetic – Pharmacodynamic variables (PK-PD integration)

Pharmacokinetic variables derived in the present study was integrated with *in vitro* pharmacodynamic measurements *i.e.*, established MIC values (Sharma *et al.*, 1994; Andersson and MacGowan, 2003; Prescott and Baggot, 1993) of norfloxacin against selected bacterial pathogens in rabbits with one or more pharmacokinetic parameters *viz*: C_{max} , AUC and duration of therapeutic plasma drug concentration found (Toutain *et al.*, 2002; McKellar *et al.*, 2004). The following three indices were used for PK – PD analysis:

- a. AUC /MIC
- b. C_{max} /MIC
- c. T > MIC

3.9.1 AUC/MIC ratio

The area under inhibitory curve (AUC) was determined according to McKellar *et al.* (2004) to predict the efficacy of norfloxacin.

$$\text{AUC} = \frac{\text{AUC}}{\text{MIC}}$$

Where, AUC = area under the curve

MIC = minimum inhibitory concentration

3.9.2 C_{max} /MIC

Norfloxacin, being a member of fluroquinolone class of antibacterial agents possess concentration dependent bactericidal effect. The ratio of C_{max} /MIC greater than 8 was considered as an index to

compare the efficacy of fluoroquinolones according to Nicolau *et al.*, (1995) and McKellar *et al.* (2004). The efficacy of norfloxacin with respect to $C_{\max}/\text{MIC}$ was compared between control (group-I) and curcumin treated (group-II) rabbits.

3.9.3 T > MIC

The minimum inhibitory concentration (MIC) maintaining 52-60 per cent of the inter dose interval is considered as an absolute requirement for drugs acting by time-dependent mechanisms only (Toutian *et al.*, 2002). Therefore, the $T > \text{MIC}$ was examined for the purpose of relative comparison between two groups.

3.10 Statistical analysis

Statistical analysis of the data was performed according to Snedecor and Cochran (1994). The plasma concentration of norfloxacin and its pharmacokinetic variables were expressed as mean $\pm$ SE. The pharmacokinetic variables between two groups were compared using Student's 't' test.

RESULTS

IV. RESULTS

4.1 Plasma concentration - time profile of norfloxacin

The plasma concentration of norfloxacin observed at different time intervals in group-I (control) and group-II (curcumin treated) rabbits is presented in Table-4. Norfloxacin was absorbed rapidly following oral administration with a mean plasma concentration of $1.50 \pm 0.25 \mu\text{g.ml}^{-1}$ and $1.08 \pm 0.03 \mu\text{g.ml}^{-1}$ at 30 minute in control and curcumin treated groups respectively. The peak plasma norfloxacin concentration (C_{max}) $2.23 \pm 0.08 \mu\text{g.ml}^{-1}$ was achieved (t_{max}) at 0.25 h in control group (Table 4). Norfloxacin concentration in control group was detected in plasma up to 12 h (Figure.4) with a mean concentration of $0.62 \mu\text{g.ml}^{-1}$.

In curcumin treated rabbits, the absorption of norfloxacin was relatively rapid and the observed values of C_{max} and t_{max} were respectively $2.87 \pm 0.17 \mu\text{g.ml}^{-1}$ and 0.16 h. The plasma norfloxacin concentration was persistent up to 24 h (Figure.5) with a mean concentration of $0.69 \mu\text{g.ml}^{-1}$. The comparative semilogarithmic plot of mean plasma concentration-time profile of norfloxacin between control and treated groups is depicted in Figure. 6

4.2 Pharmacokinetics of norfloxacin in control and curcumin treated rabbits

The plasma concentration-time profile norfloxacin in rabbits after single oral administration @ 100 mg/kg was examined individually after semilogarithmic plot. The plasma disposition kinetics of norfloxacin was best described by one-compartment open model by virtue of mono-exponential decay after single oral administration in control and curcumin treated animals.

Table-4: Plasma concentration of norfloxacin in control (group-I) and curcumin pre-treated (group-II) rabbits following single oral administration @ 100 mg/kg (mean $\pm$ SE)

Time(h)	Group-I (Control) n=8 $\mu\text{g/ml}$	Group-II (Curcumin treated) n=8 $\mu\text{g/ml}$
0.08	0.37 $\pm$ 0.05	0.50 $\pm$ 0.25
0.16	0.46 $\pm$ 0.04	2.87 $\pm$ 0.17
0.25	2.23 $\pm$ 0.08	1.19 $\pm$ 0.01
0.50	1.50 $\pm$ 0.25	1.08 $\pm$ 0.03
0.75	0.63 $\pm$ 0.04	0.97 $\pm$ 0.04
1.00	0.62 $\pm$ 0.01	0.84 $\pm$ 0.01
1.15	0.59 $\pm$ 0.01	0.74 $\pm$ 0.02
1.30	0.53 $\pm$ 0.04	0.71 $\pm$ 0.09
2.00	0.48 $\pm$ 0.03	0.59 $\pm$ 0.01
3.00	0.46 $\pm$ 0.01	0.48 $\pm$ 0.04
4.00	0.41 $\pm$ 0.01	0.32 $\pm$ 0.03
5.00	0.39 $\pm$ 0.01	0.29 $\pm$ 0.01
6.00	0.37 $\pm$ 0.01	0.21 $\pm$ 0.06
8.00	0.17 $\pm$ 0.04	0.13 $\pm$ 0.01
12.00	0.015 $\pm$ 0.001	0.09 $\pm$ 0.01
24.00	ND	0.07 $\pm$ 0.02

Note: ND- Not detected

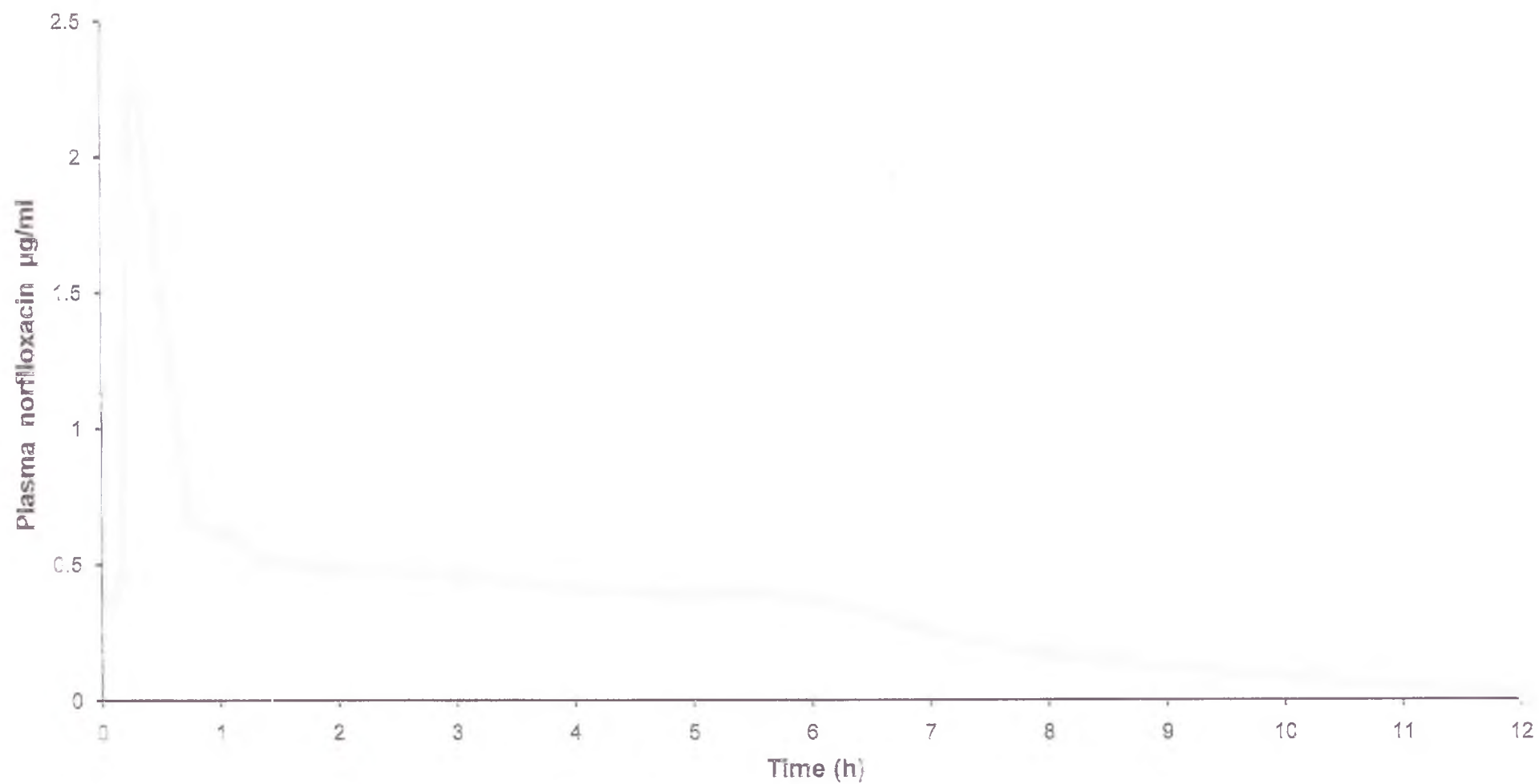


Fig.4: Mean plasma concentration-time profile of norfloxacin in rabbits (Group-I) following a single per-oral dose (100mg/Kg; n=8)

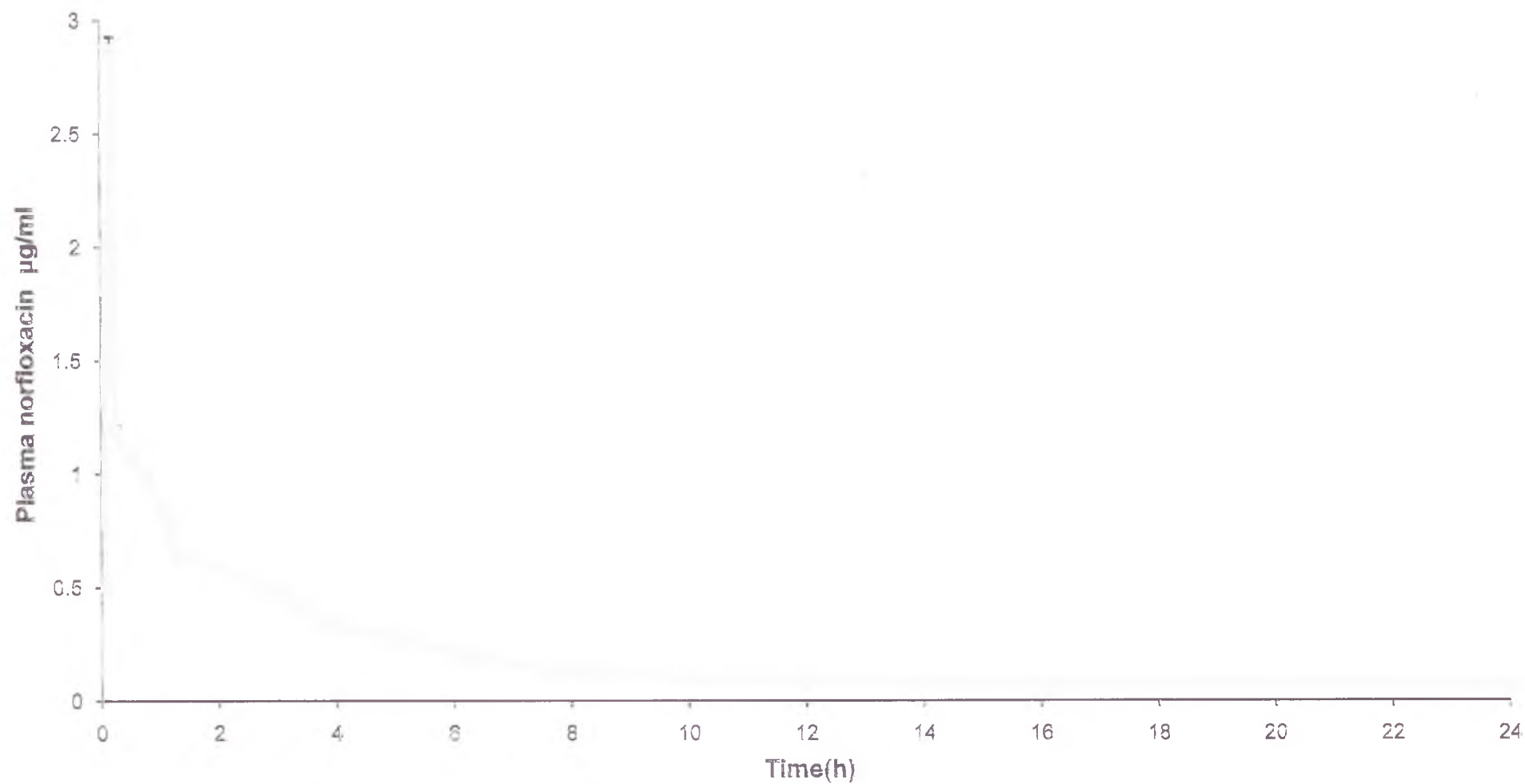


Fig. 5: Mean plasma concentration- time profile of norfloxacin in rabbits pretreated with curcumin(Group-II) following a single per-oral dose (100mg/Kg; n=8)

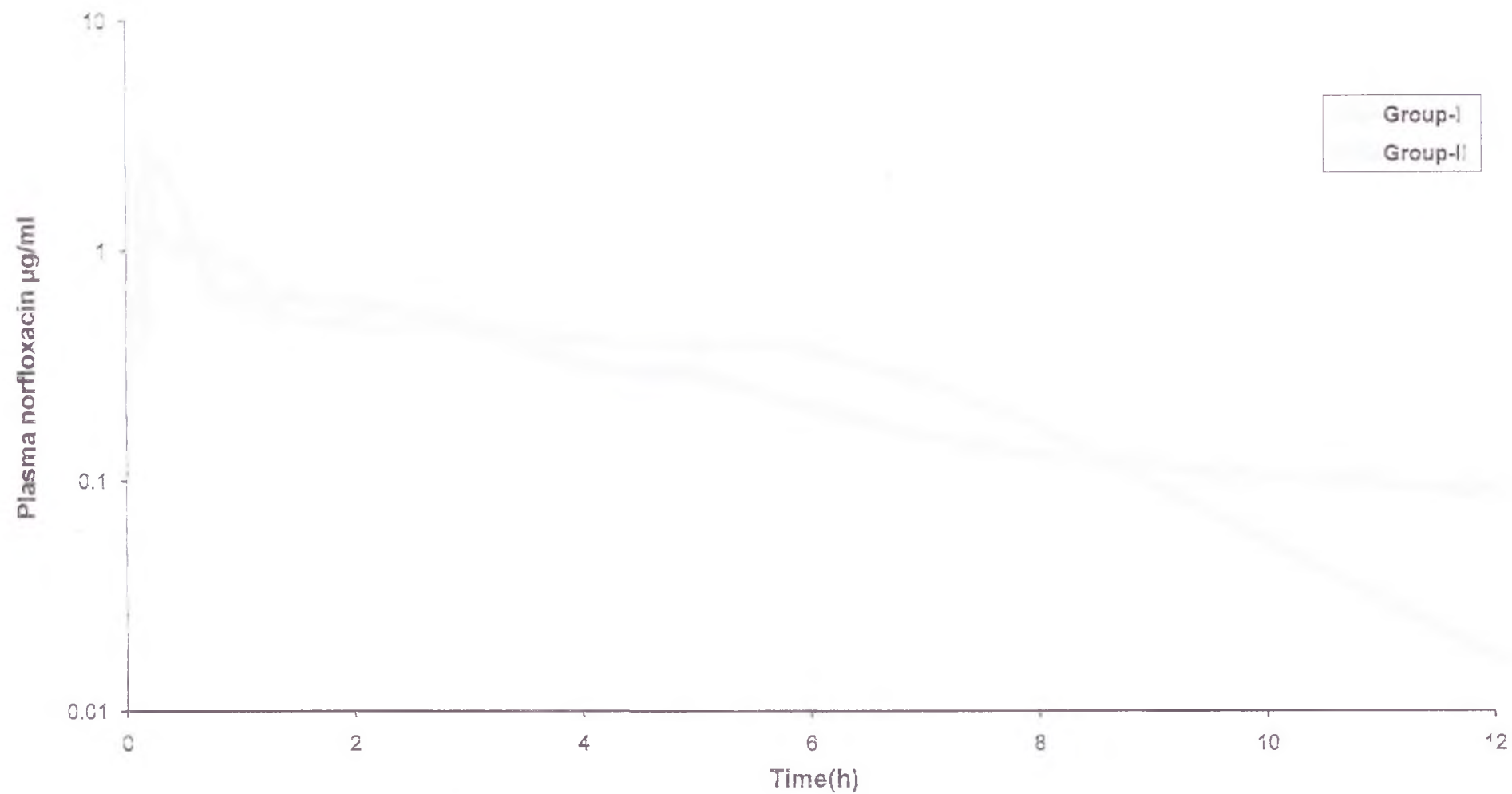


Fig.6: Semilogarithmic plot of mean plasma concentration-time profile of norfloxacin in control (Group-I) and curcumin pre-treated rabbits (Group-II) following a single per-oral dose

The pharmacokinetic parameters for individual rabbits belonging to two groups were calculated. The comparative mean pharmacokinetic variables are presented in Table-5. The absorption rate constant (K_a) between control ($1.84 \pm 0.03 \text{ h}^{-1}$) and curcumin treated ($2.59 \pm 0.62 \text{ h}^{-1}$) rabbits differed significantly ($P \leq 0.05$). The absorption half-lives ($t_{1/2K_a}$) in curcumin treated animals were relatively short.

Pre-treatment of rabbits with curcumin modified the kinetic profile of norfloxacin as evidenced by significantly ($P \leq 0.05$) higher plasma-elimination half-life ($t_{1/2\beta}$), AUC, AUMC, $V_{d(\text{area})}$. The $t_{1/2\beta}$, AUC, AUMC and $V_{d(\text{area})}$ were $2.49 \pm 0.13 \text{ v/s } 2.96 \pm 0.34 \text{ h}$, $2.67 \pm 0.42 \text{ v/s } 4.06 \pm 1.24 \mu\text{g.h.ml}^{-1}$, $13.40 \pm 1.62 \text{ v/s } 22.64 \pm 6.34 \mu\text{g.h}^2.\text{ml}^{-1}$ and $5.69 \pm 0.28 \text{ v/s } 7.45 \pm 1.70 \text{ L.kg}^{-1}$ in control and curcumin treated rabbits respectively (Table-5).

The MRT of norfloxacin in group-II was significantly ($P \leq 0.05$) higher when compared to control group. The total body clearance rate between two groups (1.49 ± 0.11 in control v/s $1.58 \pm 0.03 \text{ L.Kg}^{-1}.\text{h}^{-1}$ in treated group) differed non-significantly ($p > 0.05$). The total duration (t_d) of pharmacological effect was relatively more in rabbits pre-treated with curcumin.

The per-oral bioavailability of norfloxacin in rabbits (group-I) was found to be 41.82 ± 6.65 per cent. Curcumin pre treatment (group-II) significantly ($P \leq 0.05$) increased the gastrointestinal absorption of norfloxacin ($64.10 \pm 18.78\%$) in rabbits (Table-6).

No untoward clinical signs or gastrointestinal disturbances were noticed in rabbits (group-II) following per-oral administration of curcumin @ $60 \text{ mg/kg} \times 3$ days.

Table-5: Comparative pharmacokinetic variables of norfloxacin after single oral administration in control (group-I) and curcumin treated (group-II) rabbits (100 mg/kg; mean $\pm$ SE; n=8)

Parameter	Unit	Group I	Group-II
K _a	h ⁻¹	1.84 $\pm$ 0.03	2.59 $\pm$ 0.62*
A	$\mu\text{g.ml}^{-1}$	1.95 $\pm$ 0.14	1.85 $\pm$ 1.15
β	h ⁻¹	0.278 $\pm$ 0.01	0.231 $\pm$ 0.03*
B	$\mu\text{g.ml}^{-1}$	1.04 $\pm$ 0.01	1.11 $\pm$ 0.02
t _{1/2 Ka}	h	0.35 $\pm$ 0.01	0.27 $\pm$ 0.06
t _{1/2β}	h	2.49 $\pm$ 0.13	2.96 $\pm$ 0.34 *
AUC	$\mu\text{g h.ml}^{-1}$	2.67 $\pm$ 0.42	4.06 $\pm$ 1.24 *#
AUMC	$\mu\text{g h}^2.\text{ml}^{-1}$	13.40 $\pm$ 1.62	22.64 $\pm$ 6.34 *#
MRT	h	5.01 $\pm$ 0.19	5.60 $\pm$ 0.15 *
V _{d(area)}	L.Kg ⁻¹	5.69 $\pm$ 0.28	7.45 $\pm$ 1.70 *
Cl _B	L.Kg ⁻¹ . h ⁻¹	1.49 $\pm$ 0.11	1.58 $\pm$ 0.03
t _d	h	27.72 $\pm$ 6.22	29.58 $\pm$ 3.84

* P $\leq$ 0.05; # P $\leq$ 0.01

NOTE: K_a = absorption rate constant
A = zero time plasma drug concentration intercept of regression line of absorption phase
 β = overall elimination rate constant
B = zero time intercept of regression line of elimination phase
t_{1/2 Ka} = absorption half life
t_{1/2 β} = elimination half life
AUC = area under curve
AUMC = area under moment of plasma drug concentration-time curve
MRT = mean residence time
t_d = total duration of pharmacological effect
Cl_B = total body clearance

Table 6 : C_{max}, t_{max} and bioavailability of norfloxacin in different groups of rabbits after single oral administration (100mg/kg, n=8)

Parameter	Unit	Group - I	Group - II
C _{max}	µg.ml ⁻¹	2.23 ± 0.08	2.87 ± 0.17*
t _{max}	h	0.25 #	0.16 #
Bioavailability(F)	%	41.82 ± 6.65	64.10 ± 18.78 *

Note: * P ≤ 0.05; # Values are harmonic means; Animals in group II were pre-treated with curcumin @ 60 mg/kg, p.o x 3 days

4.3 Dosage regimen

The dosage regimen of norfloxacin required to maintain desired MIC (0.1, 0.5 and 1.0 $\mu\text{g/ml}$) in rabbits is tabulated in Table-7. Practically in order to maintain MIC 0.5 $\mu\text{g.ml}^{-1}$ in plasma, norfloxacin has to be administered (p.o) at the rate of 15, 26 and 80 mg/kg as loading dose followed by 12, 24 and 77 mg/kg as maintenance dose respectively at 6, 8 and 12 h intervals in rabbits.

Curcumin pre-treatment in rabbits was found to be beneficial, in terms of dose required to maintain desired MIC values of norfloxacin was relatively less. Administration of norfloxacin (p.o) at an interval of 12 h is desirable from practical point of view. Therefore, pre-treatment with curcumin followed by norfloxacin @ 12, 60 and 120 mg/kg as loading dose and respectively 11, 55 and 110 mg/kg as maintenance dose is required to be administered twice daily in rabbits. Thus, the computed dosage regimen ensures MIC of 0.1, 0.5 and 1.0 $\mu\text{g.ml}^{-1}$ plasma respectively. Pre-treatment with curcumin reduced the loading and maintenance dose of norfloxacin (12 h interval, 0.5 $\mu\text{g.ml}^{-1}$ MIC) by 26 and 24 per cent respectively.

4.4 Pharmacokinetic-pharmacodynamic (PK-PD) integration

The PK-PD integration using the pharmacokinetic variables derived in the present study was computed to test and optimize the dosage regimen of norfloxacin either given alone or after curcumin pre-treatment. The PK-PD integration of norfloxacin against important bacterial pathogens and *Mycoplasma pneumoniae* is tabulated in Table-8.

The area under the inhibitory curve (AUC) calculated as the ratio of $\text{AUC}_{(0-24)}/\text{MIC}$ showed excellent (>125) antibacterial activity against *Pasteurella multocida* and mycoplasmacidal effect on *Mycoplasma*

Table 7: Loading and maintenance dose (mg.Kg⁻¹) of norfloxacin in rabbits pre-treated with or without curcumin

Desired MIC ($\mu\text{g.ml}^{-1}$)	Group – I (Norfloxacin) Dosage interval			Group – II (Curcumin* + Norfloxacin) Dosage interval		
	6h	8h	12h	6h	8h	12h
0.1	3.01 (2.45)	5.25 (4.69)	15.99 (15.42)	2.97 (2.23)	4.73 (3.98)	11.91 (11.17)
0.5	15.08 (12.23)	26.29 (23.45)	79.96 (77.12)	14.89 (11.20)	23.64 (19.92)	59.56 (55.84)
1.0	30.16 (24.47)	52.59 (46.90)	159.92 (77.11)	29.79 (22.34)	47.29 (39.84)	119.13 (111.68)

Note : *Curcumin was administered @ 60 mg/kg x 3 days, p.o; Values given in parenthesis are maintenance doses; Dosage regimen was derived as per the method described by Notari, 1987

Table-8: PK-PD integration of norfloxacin against important bacterial pathogens and mycoplasma in rabbits

Pathogen	MIC₉₀ ($\mu\text{g} \cdot \text{ml}^{-1}$)	PK-PD integration (using PK data derived in the present study)			
		Control (group I)		Curcumin @ treated (group-II)	
		AUIC	C_{max}/MIC	AUIC	C_{max}/MIC
<i>P. multocida</i>	0.02 *	133.50	111.50	203.0	143.50
<i>Salmonella</i> spp.	0.03*	89.00	74.33	135.00	95.66
<i>E.coli</i>	0.03*	89.00	74.33	135.00	95.66
<i>Haemophilus</i> spp.	0.06#	44.50	37.16	67.16	47.83
<i>Mycoplasma</i> <i>pneumoniae</i>	0.02*	133.50	111.50	203.00	143.50

Note: * Prescott and Baggot (1993); # Andersson and MacGowan (2003);
@ 60 mg/kg x 3 days, p.o

pneumoniae. Further, the ratio was found to be one-and half times higher in rabbits pre-treated with curcumin. Similarly, curcumin pre-treatment has markedly improved the AUC of norfloxacin against *Salmonella* spp., *E.coli* and *Haemophilus* spp.

The ratio of C_{max}/MIC was optimal (>10) in both groups of rabbits, however, the ratio was relatively higher in rabbits treated with curcumin. The C_{max} was approximately 50-150 fold higher (group-II) than the MIC of norfloxacin. However, PK PD integration of data revealed that norfloxacin alone or after curcumin treatment failed to satisfy the prescribed AUC and C_{max}/MIC ratio with respect to *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella pneumoniae* (data not shown in the Table-8).

The minimum therapeutic plasma level ($\geq 0.02 \mu\text{g.ml}^{-1}$) up to 12 and 24 h was observed respectively in control and curcumin treated group following single per oral administration of norfloxacin.

DISCUSSION

V. DISCUSSION

Pharmacokinetics is the mathematical description of concentration changes of a given drug or its metabolite(s) as a function of time in the body. The study of pharmacokinetics is primarily based on the creation of mathematical models to describe and predict the drug concentration in the body (Riegelman *et al.*, 1968). Thus, pharmacokinetic analysis is helpful to ensure safety and optimisation of dosage schedule.

Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid] is a fluorinated quinolone which has broad spectrum of antibacterial activity mediated by affecting bacterial DNA-*gyrase*. The broad objectives of the present study was to determine the pharmacokinetic profile of norfloxacin following *per-os* administration and secondly, to understand the influence of curcumin (an active principle of *Curcuma longa* L.) pre-treatment on disposition kinetics of norfloxacin in rabbits.

5.1 Analysis of PK data by one-compartment open model

The plasma concentration-time profile of norfloxacin after single oral dose of norfloxacin in the present study was best described by one-compartment open model following single oral administration at a dose level of 100 mg/kg. Similar dose (*per os*) has been used to describe plasma and tissue concentration of norfloxacin in rabbits (Rylander and Norrby, 1983). One-compartment open model depicts the body as a single homogenous unit. This model is particularly useful for drugs which rapidly distributes between plasma and other body fluids and tissues upon entry into systemic circulation (Venkateswarlu and Kokate, 2000). Single-compartment has been employed to describe the disposition kinetics of pefloxacin in goats (Madhukar, 2006), oxytetracycline in hens (Singh *et al.*, 2005), ampicillin and amoxicillin in

buffaloes (Khanikor *et al.*, 1986) after extra-vascular administration. However, two-compartment open model was used to describe pharmacokinetics of norfloxacin and its metabolite(s) in broiler chickens (Anadon *et al.*, 1992) or norfloxacin glycine acetate in swine (Chang *et al.*, 2007) after oral or intravenous administration.

5.2 Pharmacokinetics of norfloxacin after single oral administration in rabbits (group-I)

The rate constant for absorption (K_a) of norfloxacin after oral administration is more than that of rate constant for elimination (β) and it was derived from slope of the residual concentration obtained by the method of residues. The absorption of norfloxacin administered in solution form was rapid after oral administration in rabbits with a C_{max} of $2.23 \pm 0.08 \mu\text{g.ml}^{-1}$ after single oral dose and the data obtained is comparable to previous studies in rabbits (Rylander and Norrby, 1983) after similar dose. However, C_{max} was relatively more when compared to previous studies in rabbits (Park *et al.*, 1988) and broiler chickens (Anadon *et al.*, 1992). The plasma concentration of norfloxacin observed at 8 h ($0.17 \pm 0.04 \mu\text{g.ml}^{-1}$; Table-4) was markedly above the minimum inhibitory concentration (MIC) of certain Gram negative and atypical bacteria (Sharma *et al.*, 1994; Andersson and MacGowan, 2003). However, the plasma concentration of norfloxacin was detected up to 12 h post-administration in rabbits ($0.015 \pm 0.001 \mu\text{g.ml}^{-1}$) maintaining the MIC against only few pathogens including *Mycoplasma pneumoniae* (Prescott and Baggot, 1993).

Bioavailability of norfloxacin after oral dose was variable between individual animals and moderate ($41.82 \pm 6.65\%$) despite the fact that rabbits were dosed after overnight fasting. Similarly, the bioavailability of norfloxacin in dogs was reported to vary significantly between individual animals ($35.0 \pm 46.10\%$) after single oral dose (Brown *et al.*, 1990). Our

results were comparable to bioavailability of norfloxacin (40%) in rabbits (Park *et al.*, 1988) but relatively less than the reported values ($57.0 \pm 2.4\%$) in broiler chickens (Anadon *et al.*, 1992). It has been reported that the systemic bioavailability of norfloxacin was only 30-40 per cent (Neuman, 1988) in most species. The absolute bioavailability of norfloxacin in humans and in laboratory animals is 40 per cent (Holmes *et al.*, 1985). The bioavailability was poor after oral administration of either norfloxacin glycine acetate (31.10%) in pigs (Chang *et al.*, 2008) or norfloxacin nicotinate ($<10.0\%$) in donkeys (Lavy *et al.*, 1995). The precise mechanism of norfloxacin absorption across the gastrointestinal (GI) tract is not known. However, GI-transit time might have influenced the norfloxacin absorption in the present study.

The area under the curve (AUC) was found to be considerably small ($2.67 \pm 0.42 \mu\text{g.h.ml}^{-1}$) despite the fact that a large dose was employed in the present study. However, the AUC of norfloxacin was reported to be less when compared to other members of the fluoroquinolone group of antibacterial agents (Andersson and MacGowan, 2003). Brown *et al.* (1990) reported that AUC per unit dose decrease linearly with dose following per oral administration of norfloxacin in random-source female dogs. The AUC is the area which is directly proportional to the total amount of drug introduced into the central compartment (blood). Therefore, the lower AUC observed in the present study may be sequelae to faster clearance from the body or due to reduced residence time in the absorptive areas of the GI-tract for absorption of the larger dose.

The rate constant of elimination of norfloxacin from plasma was slower than the rate of absorption ($K_a=1.84 \text{ h}^{-1}$, $\beta=0.278 \text{ h}^{-1}$; Table-5). The elimination rate constant characterise the overall elimination from one-compartment by renal and biliary excretion. Norfloxacin undergoes

excretion both by metabolism and renal mechanisms (Sharma *et al.*, 1994; Rao and Tripathi, 1999). Although a detailed comparative metabolic profile of norfloxacin is not known, five metabolites of norfloxacin have been identified among which the major metabolites includes oxo-norfloxacin and desethylene-norfloxacin (Anadon *et al.*, 1992). However, the plasma half-life of norfloxacin was reported to be short as compared to new generation fluoroquinolones (Andersson and MacGowan, 2003). The plasma half-life ($t_{1/2\beta}$) of norfloxacin in the present study was 2.49 ± 0.13 h and therefore it is evident that the time required to reach steady state will be relatively more since generally four-to-five half-lives are required to achieve that (Nicolau *et al.*, 1995).

The half-lives of norfloxacin observed in the previous studies in rabbits (3.93 h; Park *et al.*, 1988); dogs (3.56 h; Brown *et al.*, 1990); man (3.3-5.5 h; Sharma *et al.* 1994) and donkeys (3.52 h; Lavy *et al.*, 1995) were comparatively more. On the contrary, the $t_{1/2\beta}$ obtained in the present study was remarkably more when compared to values in laboratory mouse (20min; Renneberg and Walder, 1989) suggesting marked difference in pharmaco-disposition of norfloxacin between species. Similarly, $t_{1/2\beta}$ of norfloxacin analogues were reported to be long in swine *viz.* norfloxacin nicotinate after i.m or s.c administration (4.45 h; Shem Tov and Ziv, 1994) and norfloxacin glycine acetate after p.o administration (7.31 h; Chang *et al.*, 2007). Thus, detailed characterization of metabolism of norfloxacin (hepatic and intestine) and its urinary and or biliary kinetics can enlighten the elimination profile in rabbits.

The volume of distribution (V_d area) was observed to be large in the current study (5.69 ± 0.28 L.Kg⁻¹; Table-5) indicate good distribution in the body. Further, $V_d > 1.0$ L.Kg⁻¹ indicates wide distribution, extensive tissue binding or both (Baggot, 1977). However, norfloxacin was relatively

less lipid soluble than pefloxacin (Delon *et al.*, 1997). Generally, fluoroquinolones are less protein bound and liposoluble drugs with a good tissue penetration and therefore possess a high V_d (Brown, 1996).

The mean residence time (MRT) obtained in the present study (5.01 ± 0.19 h) was comparable to observation made in dogs (6.38 ± 3.10 h; Brown *et al.*, 1990). However, a prolonged MRT of norfloxacin was observed in poultry (14.4 h; Pant *et al.*, 2005) or pefloxacin (which get metabolised into norfloxacin in pigeons, 10.10 h; Moutafchieva, 1997) after oral administration. Thus, norfloxacin has lesser stasis in rabbits as compared to chicken and this property is utmost important from food residue point of view.

The body clearance (Cl_B) of norfloxacin was faster which might be due to involvement of both renal and biliary route as in the case of other laboratory animals (Gilfillan *et al.*, 1984). The clearance of norfloxacin was also found to be rapid in chickens ($10.67 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$; Anadon *et al.*, 1992). It has been reported that most of the absorbed dose is excreted as unchanged in the urine (Holmes *et al.*, 1985).

5.3 Influence of curcumin on disposition kinetics of norfloxacin (group-II)

In the present study, the effect of curcumin (diferuloylmethane, an yellow coloured phenolic pigment present in *Curcuma longa* L.) on disposition profile of norfloxacin was examined. To our knowledge there are no previous attempts made to assess the influence of curcumin (if, any) exposure on pharmacokinetic behaviour of antimicrobials or any other therapeutic agent(s) comprehensively. However, the selection of dose and duration of treatment with curcumin in the present study (60 mg/Kg; p.o x 3days) was based on the recent report where in curcumin administration increased absorption of celiprolol following oral administration in rats (Wenxia *et al.*, 2006).

Norfloxacin was detected in plasma up to 24 h post-administration in all the rabbits which were pre-treated with curcumin. Similarly, the $C_{\max}$ ($2.87 \pm 0.17 \mu\text{g.ml}^{-1}$; Fig.5) and the overall mean plasma concentration ($0.685 \mu\text{g.ml}^{-1}$) were also higher than that of rabbits in group-I. The absorption rate (K_a) constant for norfloxacin was significantly ($P \leq 0.05$) higher in group-II however, the difference in absorption half-life ($t_{1/2K_a}$) was non-significant ($P \geq 0.05$; Table-5). Further, the bioavailability of norfloxacin was significantly ($P \leq 0.05$; Table-6) more after curcumin pre-treatment in rabbits. Thus, it was evident from the present study that curcumin exposure influence the absorption phase or mechanism(s) involved in absorption of norfloxacin from GI-tract. The increased bioavailability of norfloxacin in rabbits (group-II) may be due to stasis of norfloxacin at the absorptive surface of small intestine relatively for a longer period due to anti-spasmodic effect by curcumin and or delay in gastric emptying time (Srihari Rao *et al.*, 1982; Chattopadhyay *et al.*, 2004). In addition to it, increased bioavailability of norfloxacin may be due to bypass of glucuronidation process in the intestine since curcumin was reported to suppress UDP-glucuronyltransferase levels in intestinal and hepatic tissue (Owens *et al.*, 2004). Similarly, curcumin has been shown to inhibit glutathione-S-transferase PI-1(GSPTI-1) expression at transcription level (Gautam *et al.*, 1998; Chattopadhyay *et al.*, 2004). However, the exact mechanism(s) involved in absorption of norfloxacin across the GI-tract was least understood.

Alternatively, it is more likely that increased bioavailability observed in the present study may be due to the ability of curcumin to influence drug transporter protein (P-gp) in the intestine as in the case of celiprolol (Wenxia *et al.*, 2006). Piperine (an alkaloid present in *Piper longum*, *Piper nigrum* and *Zingiber officinalis*) is a well known inhibitor of P-gp protein (Baradwaj *et al.*, 2002) and thereby modify the bioavailability of drugs. Co-administration of piperine has been shown to

increase bioavailability of enrofloxacin and its metabolite ciprofloxacin in goats (Ramesh, 2001) and β -lactam antibiotics in rats (Hiwale *et al.*, 2002). Similarly, one week oral exposure to *Piper longum* (equivalent to 15 mg) has improved the absorption of oxytetracycline after oral administration in hens (Singh *et al.*, 2005). Therefore, it is not unfair to correlate the increased bioavailability of norfloxacin observed in the present study (group-II) with the altered drug absorption mechanism(s) consequent to inhibition of P-gp protein.

It is interesting to note that the disposition kinetics of norfloxacin was modified in rabbits after pre treatment with curcumin (group-II; Table-5) in a manner which can be exploited for clinical advantage. Curcumin pre-treatment (@ 60 mg.Kg⁻¹ x 3days; p.o) has significantly increased the AUC ($P \leq 0.01$; $P \leq 0.05$), AUMC ($P \leq 0.01$; $P \leq 0.05$), MRT ($P \leq 0.05$), $t_{1/2\beta}$ ($P \leq 0.05$) and $V_{d(\text{area})}$ ($P \leq 0.05$) of norfloxacin and exerted relatively a long duration of action (t_d) in rabbits after single oral administration.

The property of curcumin to inhibit non-specific drug metabolising enzymes and inducer of glucuronyltransferase is a well established fact. Norfloxacin undergoes extensive metabolism in the body and at least five metabolites of norfloxacin with modifications to the piperazine ring have been identified in most species *viz*: oxo norfloxacin, desethylene-norfloxacin, amino-norfloxacin, acetyl-norfloxacin and formyl-norfloxacin (Anadon *et al.*, 1992). Therefore, significantly higher values of AUC, AUMC and MRT observed in the present study can be attributable to enhanced systemic availability of norfloxacin consequent to inhibition of enzymes mostly concerned with Phase-I metabolism of norfloxacin.

Further, in contrast to the fact that curcumin can induce glucuronyltransferase, its suppression at higher dose can not be ruled

out. It is worthwhile to note that curcumin itself undergoes metabolism through hepatocytes as glucuronides of tetrahydrocurcumin (Pan *et al.*, 1999) and therefore, the metabolism of norfloxacin may get delayed due to competition between two substrates.

The observation made in the present study can be comparable to the studies in which piperine has been employed to enhance therapeutic effect of drugs. Piperine was also found to be a non-specific inhibitor of drug metabolising enzymes including 3-methylcholanthrene and phenobarbitol induced microsomal enzymes (Atal *et al.*, 1985) and CYP3A4 (Bharadwaj *et al.*, 2002). Singh *et al.* (2005) observed higher values of AUC, AUMC, MRT, $t_{1/2\beta}$ of oxytetracycline in hens pre-treated with *Piper longum* (equivalent to 15 mg piperine) for a week. Similar findings with respect to pefloxacin was also reported in goats (Madhukar, 2006) treated with *Trikatu*®, a commercial formulation essentially containing piperine as active ingredient.

The elimination rate constant for norfloxacin in curcumin treated rabbits was significantly ($P \leq 0.05$) low (0.278 h^{-1} in group-I v/s 0.231 h^{-1} in group-II) resulting in higher plasma elimination half-life ($t_{1/2\beta} = 2.96 \pm 0.34 \text{ h}$) when compared to control. The increase in $t_{1/2\beta}$ could be due to prolonged persistence of drug (Fig. 5 & 6) in the body due to inhibition of one or more enzyme(s) concerned with metabolism of norfloxacin. The $V_{d(\text{area})}$ of norfloxacin was significantly more in curcumin treated rabbits but the total body clearance of norfloxacin (Cl_B) was found to be statistically non-significant ($P \geq 0.05$) between two groups. A significant amount of norfloxacin was observed to be excreted as unchanged via renal mechanisms (Holmes *et al.*, 1985; Rao and Tripathi, 1999) and therefore the ability of curcumin to delay excretory mechanism at the proximal convoluted tubules can not be ruled out since P-gp protein also exists there.

5.4 Dosage regimen and PK-PD interaction of norfloxacin

The pharmacodynamic (PD) properties or the bactericidal effect of a specific class of antimicrobials which can be best described by integrating microbiological activity in terms of minimum bactericidal concentration (MBC) or minimum inhibitory concentration (MIC) with pharmacokinetic properties (Nicolau *et al.*, 1995; Toutain *et al.*, 2002). Therefore, optimization of dosage of an antimicrobial agent can be determined with equation constructed by Toutain *et al.* (2002) which is related to PK-PD parameters. Further, Schentag (1999) observed that the AUIC/MIC ratio (=AUIC) of quinolones should be more than 125 to prevent selection pressure that leads to overgrowth of resistant bacterial sub-population.

The plasma concentration of norfloxacin persisted up to 12, 24 h post-administration in control and curcumin treated rabbits (Fig. 4 and 5). MIC of norfloxacin against common pathogens of rabbits are considerably high compared to new generation fluoroquinolones. In the present study, relatively short $t_{1/2\beta}$ and moderate level of bioavailability were observed in rabbits (group-I). However, curcumin pre treatment (group-II) significantly ($P \leq 0.05$) enhanced these parameters. Norfloxacin being a member of fluoroquinolone group of antimicrobials exert post-antibiotic effect (PAE) to the extent of three to four hours against Gram-negative or Gram-positive organisms (Renneberg and Walder, 1989). However once daily dosing regimen in rabbit is practically ineffective despite a short $t_{1/2\beta}$ is not an absolute pre-requisite for once daily dosing, since quinolones possess long PAE (Gootz and Brighty, 1996). Pharmacokinetic pharmacodynamic integration of data revealed oral administration of norfloxacin ensures desirable AUIC or $C_{\max} / \text{MIC}$ ratio. When MIC against common pathogens of rabbits are in the range of 0.01-0.03 $\mu\text{g.ml}^{-1}$ (Table 8). Therefore, administration of norfloxacin (p.o) is not ideal in rabbits suffering from infections due to Gram-positive

organisms or drug resistant Gram-negative strains whose MIC will be invariably high. Prescott and Yielding (1990) observed that norfloxacin is not an ideal drug to treat infections in pigs caused by *Streptococcus* spp., (MIC; 6.25 $\mu\text{g.ml}^{-1}$), *Staphylococcal* spp., (MIC; 1.56 $\mu\text{g.ml}^{-1}$) and any other organism whose MIC values is $>0.25 \mu\text{g.ml}^{-1}$. Therefore, from practical point of view, a dosage regimen of 80 and 77 mg/Kg of norfloxacin alone or 60 and 55 mg/kg of norfloxacin after curcumin pre-treatment as loading and maintenance dose respectively at 12 h interval adequately maintains effective MIC $0.5 \mu\text{g.ml}^{-1}$ plasma in rabbits.

The high free-drug C_{max} combined with lower MIC are of significant advantage because it increases $C_{\text{max}} / \text{MIC}$ ratio. The protein binding of norfloxacin was reported to be <30 per cent (Andersson and MacGowan, 2003). McKellar *et al.* (2004) reported that $C_{\text{max}} / \text{MIC}$ ratio >8 is essential for fluoroquinolones to exert good bactericidal effect. In the present study administration (p.o) of norfloxacin satisfies the said criteria against pathogens whose $\text{MIC} \leq 0.25 \mu\text{g.ml}^{-1}$. Similarly, curcumin pre treatment has increased efficacy of norfloxacin against pathogens whose $\text{MIC} \leq 0.35 \mu\text{g.ml}^{-1}$ in addition to reduction in loading and maintenance dose respectively.

SUMMARY

VI. SUMMARY

Norfloxacin (1 ethyl-6 fluoro-1,4-dihydro -4- oxo-7-(1-piperazinyl) 3-quinolone carboxylic acid), a member of fluroquinolone group of synthetic antibacterial agent employed in veterinary medicine. The present study was carried out in adult rabbits with the objectives *viz*: a) to establish pharmacokinetic variables of norfloxacin after single per-oral administration and to derive suitable dosage regimen b) -to study the influence of curcumin (*Curcuma longa* L.) pre-treatment on disposition kinetics of norfloxacin and c) -to derive pharmacokinetic-pharmacodynamic (PK-PD) interactions between norfloxacin and important pathogens of rabbits.

Sixteen adult New Zealand white rabbits of either sex maintained under normal laboratory conditions were divided into two groups of eight each. Rabbits in group-I (control) received norfloxacin @ 100 mg/kg, as a single pre-oral dose. Rabbits in group-II (treatment) also received similar dose of norfloxacin but after pre-treatment with curcumin (Sigma, St. Luis, MO, USA) @ 60 mg/kg x 3 days given at 24 h interval. Blood samples were drawn from the marginal ear vein into heparin coated tubes at 0, 5, 10, 15, 30, 45 min and 1, 1.15, 1.30, 2, 3, 4, 5, 6, 8, 12 and 24 h post administration.

Norfloxacin was extracted from plasma by using liquid-liquid extraction technique using acetonitrile. Quantification of norfloxacin was carried out by injecting 10 µl of each sample into a high performance liquid chromatography (HPLC, Shimadzu, Japan) system equipped with a C₁₈ reverse phase column (Phenomenox®) and measured at a UV wavelength of 275 nm. The mobile phase was composed of 0.1 per cent orthophosphoric acid and acetonitrile (850:150 v/v; pH 2.0) and delivered at a flow rate of 1ml/min. The validated limit of norfloxacin

detection by HPLC was 0.015 $\mu\text{g.ml}^{-1}$. The extraction recovery was 94.17 per cent and the intra- and inter-day assay co efficient of variation were less than 10 per cent indicating high reproducibility.

The plasma concentration-time profile of norfloxacin of individual rabbit was used to determine the pharmacokinetics of norfloxacin. Different pharmacokinetic parameters were derived after compartment analysis using mono-exponential equation: $C_p = B e^{-\beta t} - A e^{-k_a t}$. The dosage regimen for maintaining MIC of 0.1, 0.5 and 1.0 $\mu\text{g.ml}^{-1}$ at the dosage interval of 6, 8 and 12 h was constructed. The PK-PD integration was carried out by utilizing the PK variables derived in the present study and the established MIC of norfloxacin against important bacterial and mycoplasma pathogens infecting rabbits.

The plasma concentration-time profile of norfloxacin was best described by one-compartment open model with first order absorption. The mean peak plasma concentration ($C_{\max}$), time at which achieved ($t_{\max}$) and bioavailability in group-I rabbits was respectively 2.23 $\mu\text{g.ml}^{-1}$, 0.25h and 41.82 per cent. The $C_{\max}$, $t_{\max}$ and bioavailability of norfloxacin in curcumin treated rabbits (group-II) was respectively 2.87 $\mu\text{g.ml}^{-1}$, 0.16h and 64.10 per cent. Statistical analysis of the data revealed significantly ($P \leq 0.05$) higher $C_{\max}$ and bioavailability of norfloxacin in rabbits pre-treated with curcumin.

The mean absorption rate constant (K_a), absorption half-life ($t_{1/2ka}$) plasma elimination half-life ($t_{1/2\beta}$), area under the curve (AUC), area under the first moment of plasma drug concentration-time curve (AUMC) and mean residence time (MRT) was respectively 1.84 h^{-1} , 0.35 h, 2.49 h, 2.67 $\mu\text{g.h.ml}^{-1}$, 13.40 $\mu\text{g.h}^2.\text{ml}^{-1}$ and 5.01 h in group-I rabbits. Pre-treatment with curcumin (group II) significantly modified the disposition kinetics of norfloxacin in rabbits as evidenced by higher AUC

(4.06 $\mu\text{g.h.ml}^{-1}$), AUMC (22.64 $\mu\text{g.h}^2.\text{ml}^{-1}$), MRT (5.60 h). Similarly, prior treatment with curcumin significantly ($P \leq 0.05$) reduced the elimination rate constant (β) and subsequently increased the plasma elimination half-life of norfloxacin in rabbits. The volume of distribution ($V_{d(\text{area})}$) and clearance from body (Cl_B) of norfloxacin were respectively 5.69 L.kg^{-1} and 1.49 $\text{L.kg}^{-1}.\text{h}^{-1}$ in control and 7.45 L.kg^{-1} and 1.58 $\text{L.kg}^{-1}.\text{h}^{-1}$ in curcumin treated rabbits.

Computation of loading and maintenance dose revealed administration (p.o) of norfloxacin at 12, 60, 120 mg/kg as loading dose and 11, 55, 110 mg.kg^{-1} as maintenance dose at 12 h interval ensures MIC of 0.1, 0.5 and 1.0 $\mu\text{g.ml}^{-1}$ plasma respectively. Prior administration of curcumin was found to be beneficial in reducing the loading and maintenance dose to sustain desired MIC of norfloxacin in rabbits.

The PK-PD integration of the data indicated excellent antibacterial activity of norfloxacin against *Pasteurella multocida* and mycoplasmacidal effect on *Mycoplasma penumoniae* as evidenced by higher area under the inhibitory curve (AUC) and $C_{\text{max}}/\text{MIC}$ ratio. Further, prior administration of curcumin markedly improved the AUC of norfloxacin against the important Gram-negative pathogens examined and *Mycoplasma pneumoniae*. From clinical point of view, prior administration of curcumin is beneficial in reducing the computed loading and maintenance dose of norfloxacin to the extent of 26 and 24 per cent respectively when administered at 12 h interval to sustain MIC of 0.5 $\mu\text{g.ml}^{-1}$ plasma in rabbits.

To conclude, the plasma concentration-time profile of norfloxacin in rabbits was adequately described by one-compartment open model. Prior exposure to curcumin was found to be beneficial in terms enhanced bioavailability, plasma half-life, MRT and $C_{\text{max}}/\text{MIC}$ ratio of norfloxacin after per-oral administration in rabbits.

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