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Correlation among the toxicity profiling (28-days repeated oral dose toxicity), toxicokinetics and tissue distribution data of ulifloxacin, the active metabolite of prulifloxacin in Wistar albino rats

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ABSTRACT

This experiment was designed to investigate correlation among 28-days repeated oral dose toxicity, toxicokinetics and tissue distribution data of ulifloxacin (active metabolite of prulifloxacin) in Wistar albino rats. Prulifloxacin was administered for 28-days in rats at 0, 100, 200, 400 mg/kg/day followed by 14-days recovery period. Simultaneously different toxicokinetic parameters and tissue distributions of ulifloxacin was examined by LC–MS/MS method. Plasma levels and tissue concentrations of ulifloxacin were increased with dose-related manner. Ulifloxacin was also distributed to many tissues, and concentration in lungs nearly equivalent to the plasma concentration. Based on these results it was concluded that long-term repeated dose of prulifloxacin may produce different blood parameters abnormality, liver damage, stomach ulcer, joint damage and dysfunction of lungs in rats which relates to high tissue distribution and accumulation of ulifloxacin in these tissues. These findings help in management of prulifloxacin induced adverse effects by appropriate dose selection in clinical practice.

1. Introduction

Prulifloxacin (PRF), [(RS)-6-fluoro-1-methyl-7-[4-(5-methyl-2-oxo-1,3-dioxolen-4-yl) methyl-1-piperazinyl]-4-oxo-4H-[1,3] thiazeto [3,2-a]quinoline-3-carboxylic acid] (CAS NO 123447 -62-1), a prodrug of ulifloxacin (ULF) [(6)-6-fluoro-1-methyl -4-oxo-7-(1-piperazinyl)-4H-[1,3] thiazeto [3,2-a] quinoline-3-carboxylic acid], is a recently developed fluoroquinolone antibacterial compound with a broad spectrum of activity (Fig. 1) (Prats et al., 2006; Thomas et al., 2009; Giannarini et al., 2009; Keam and Perry, 2004). PRF contains a quinolone skeleton with a four-member ring in the 1, 2 positions including a sulfur atom to increase antibacterial activity and an oxodioxolenylmethyl group in the 7-piperazine ring to improve oral absorption. ULF have been shown to target DNA gyrase and topoisomerase IV, the type II bacterial topoisomerases (Gellert et al., 1977; Khodursky et al., 1995; Hussy et al., 1986) to

Abbreviations: FDA, Food and Drug Administration; CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals; IS, internal standard; LOD, limit of detection; LLOQ, limit of quantification; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ULF, ulifloxacin; PRF, prulifloxacin; LQC, low quality control; MQC, mid quality control; HQC, high quality control; API, atmospheric pressure ionization.

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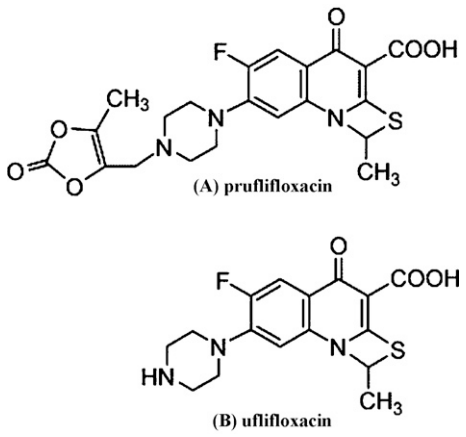

Fig. 1 – Chemical structure of (A) prulifloxacin and (B) ulifloxacin.

give activity against infections caused by a variety of clinical isolates of Gram-positive and Gram-negative bacteria (Keam and Perry, 2004; Matera, 2006). After oral administration, PRF is absorbed in the upper small intestine and then metabolized to ULF, by esterases, mainly paraoxonase enzyme, partly in intestinal membrane and mostly in portal blood and liver (Keam and Perry, 2004; Tougou et al., 1998). For this reason, PRF is not detectable in systemic circulation (Wang et al., 2007). PRF is extensively distributed and shows good penetration into different body tissues (Guillem et al., 2006; Giannarini et al., 2009). Tissue distribution of PRF into human lung tissue after oral administration has been studied by Ercole et al. (2005) and they concluded that ULF concentration in lung tissue exceeded plasma concentration at every time point. Unchanged ULF is predominantly eliminated by renal excretion (Guillem et al., 2006; Giannarini et al., 2009).

Recently a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated to determine ULF, in rat and rabbit plasma (Roy et al., 2010a,b). We have validated this developed method in different tissues of rat before the present study, because recovery and matrix effect could be different from plasma and depending on the nature of the tissue.

Limited numbers of the literatures are available on tolerability and adverse reaction profile of PRF. Adverse reaction profile is similar to that of older members of this class (Blasi et al., 2006). The pattern and incidence (around 10–15%) of adverse reaction were similar in PRF and ciprofloxacin treated patients, and mainly related to gastrointestinal disturbances with pain (Grassia et al., 2002). One older member of this group, gemifloxacin produces degenerative changes in tenocytes of Achilles tendon and erosion of the articular surface of talar joint in rats (Bae et al., 2006; Roy et al., 2010a,b). For this reason we were performed the histopathological observation of Achilles tendon, talar joint and synovial fluid analysis of talar joint of rats. A photoallergenicity study of PRF in guinea pigs was carried out by Kamata et al., 1996 and they concluded that photoallergenicity of PRF were less severe than those of the other quinolone antibacterial drugs under the conditions of their study. Prats et al. (2006) report on some data regarding phototoxicity of PRF. Older members of this group, gemifloxacin produce phototoxicity even after 7 days treatment in humans and Wistar albino rats (Roy et al., 2010a,b). From this point of view we introduced phototoxicity study in our present study. Two studies address the potential cardiotoxicity of PRF (Lacroix et al., 2003; Akita et al., 2004) and both these studies show negligible effects on cardiac depolarization/repolarization cycle *in vitro* and *in vivo* indicating a very low probability of Qc interval prolongation. Subchronic toxicity of PRF through oral route in rat and dogs has been studied by few investigators (Yoshida et al., 1996; Nishimura et al., 1996; Oda et al., 1996). However, no such studies were reported to investigate the correlation between 28-days repeated oral dose toxicity, toxicokinetics and tissue distribution data of PRF in Wistar albino rats obtaining in same experiment. Therefore, quantitative risk assessment of PRF has been hampered by the lack of sufficient data sets from animal studies. Aim of the present study was to determine the potential repeated dose toxicity, toxicokinetics and tissue distribution of ULF during 28-days oral administration of PRF, the reversibility of its toxic effects as well as tissue accumulation of ULF after recovery period of 14-days, and then extrapolated the results to investigate the correlation between oral dose toxicity, toxicokinetics and tissue distribution of ULF in Wistar albino rats in same experiment.

2. Materials and methods

2.1. *Animals' husbandry and maintenance*

Seventy-two Wistar albino rats of each sex were obtained from the animal house of Indian Institute of Chemical Biology (IICB), Kolkata, India at 6 weeks of age. These were grouped and housed in wire cages with not more than six animals per cage, under good laboratory conditions (temperature $25 \pm 2\,^{\circ}C$; $50 \pm 20\%$ relative humidity) with dark and light cycle (12/12) for minimum of 7 days before the beginning of experiment to adjust in the new environment and to overcome stress possibly incurred during transit. Only healthy animals were assigned for the study. During this period they had free access to standard dry pellet diet (Hindustan Liver, Kolkata, India) and water *ad libitum*. This study was approved by Institutional Animal Ethics Committee of Jadavpur University (CPCSEA, Reg. No. 367), Kolkata, India. Animals were maintained in accordance with the "Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA)" guide lines.

2.2. *Test article and treatment protocol*

Prulifloxacin (purity $\geq 99\%$) and ulifloxacin (purity $\geq 98\%$) bulk drugs were obtained from Cosmas Pharmaceuticals (Ludhiana, Punjab, India). Nobel Healthcare (Haryana, India) supplied Ciprofloxacin (purity $\geq 99\%$) bulk drug for analysis. Formic acid (98%) (Analytical-reagent grade) and methanol (HPLC-grade) were purchased from Merck Pvt. Ltd. (Mumbai, India). HPLC grade water (resistivity of 18.2 MΩ cm) generated from Milli Q water purification system (Elix, Milli Q A10 Academic, Molsheim, France) was used throughout the analysis. All biochemical kits obtained from Merck Pvt. Ltd., Mumbai, India.

Anticoagulant (EDTA-2K) and other reagents used were of analytical grade (Merck Pvt. Ltd., Mumbai, India). 4% PRF suspension (W/V) was prepared everyday in sterile water, and diluted by physiological saline before use. Volume of administration for rats was 10 mL/kg.

2.3. Experimental design

Dose level was selected according to the preliminary acute toxicity studies (LD50 study) in rats and dose translation from human therapeutic dose based on body surface area (BSA) (Shaw et al., 2008; Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, 2002). In preliminary acute toxicity study in rats (unpublished data), LD50 of PRF administered by oral route was about 5000 mg/kg. Human therapeutic oral dose of PRF in adult human is 600 mg/day. Based on BSA of rat oral therapeutic dose was calculated nearly 60 mg/kg. In our study, 400 mg/kg/day was selected for the high oral dose which is nearly 6.5 times of therapeutic dose and 1/12.5 times of oral LD50 dose. Doses of 200 and 100 mg/kg/day were selected as middle and low oral dose for rat, respectively, using a common ratio of 2. Dose conversation was done according to following formula (Shaw et al., 2008; Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers, Food and Drug Administration, 2002):

$$\text{Animal dose (mg/kg)} = \frac{[\text{human equivalent dose (mg/kg)} \times \text{human } K_m]}{\text{animal } K_m}$$

(K_m : surface area to weight ratios)

Present study was performed according to the "Guidelines for Repeated Dose 28-Day Oral Toxicity Study in Rodents (1995; No. 407)" provided by the Organization for Economic Co-operation and Development (OECD) and was conducted in compliance with FDA, Good laboratory practice Regulations (Part 58 of 21 Code of Federal Regulations (CFR)). Repeated dose toxicity study included both a 28-days treatment period and a 14-days recovery period.

Healthy adult male and female rats weighing 150 ± 15 g were randomly assigned to four experimental groups: PRF 100, 200, and 400 mg/kg/day groups and a vehicle control group. Each group consisted 12 (low- and middle-dose groups) and 18 (vehicle control and high-dose groups) rats of each sex. After treatment for 28-days, 12 rats per sex per group were anesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg) and were sacrificed by cervical decapitation for pathological examination. Animals of recovery groups were observed for reversibility, persistence and delayed occurrence of toxic effects. General clinical appearances and mortality of all groups were observed on both daily and weekly basis. Hematology, serum biochemistry, synovial fluid biochemistry, urine, organ weight, serum electrolyte, microscopic examination of synovial fluid and histopathology of rats were examined on day-28 and -42. Food and water consumption of all groups were also examined on weekly basis. Phototoxicity test were also performed on weekly basis within treatment period and 14th day of recovery period.

2.4. Clinical observation and mortality

Throughout the study period, all animals were observed daily for clinical signs of toxicity, morbidity and mortality.

2.5. Body weight trends

Body weights of each rat were measured at the initiation of treatment and once a week throughout the treatment and recovery period.

2.6. Food and water consumption

Food and water consumption for a group of twelve rats in two cages (six rats in each cage) were measured at the start of treatment, weekly throughout the treatment and recovery period. Amount of food and water consumption were calculated before they were supplied to each cage and their remnants were measured next day to calculate the differences, which were regards as daily food and water consumption (g/rat/day).

2.7. Urinalysis

During the last week of treatment, urinalysis of twelve males and females per group was conducted with fresh urine to identify specific gravity, pH, protein, glucose, ketone body, occult blood, bilirubin, urobilinogen and nitrite by using a CliniTek-100 urine chemistry analyzer (Ames Division, Miles Laboratory, USA). Urine sediment test was also carried out within 3 h after taking samples during the last week of administration period. Volume of urine collected for 24 h was measured. During the collection, rats were housed in metabolic cages that allowed for separate collection of urine and feces.

2.8. Hematology

Animals were fasted overnight prior to necropsy and blood collection. Blood samples were collected into tube containing EDTA-2K (Merck Pvt. Ltd., Mumbai, India) and analyzed within 1 h of collection. Hematological parameters including red blood cell (RBC) count, hemoglobin (HB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), reticulocytes (Rt), white blood count (WBC) and WBC differential counts (lymphocyte, monocyte and granulocyte) were examined by Medonic CA-620 cell analyzer systems (Boule Medical, Stockholm, Sweden).

2.9. Serum biochemistry

Blood for clinical chemistry was placed in tubes devoid of anticoagulant, allowed to clot at room temperature followed by centrifugation at 3000 rpm for 10 min within 1 h of collection, and then serum was separated. Serum biochemistry parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine phosphokinase (CPK), glucose (GLU), total protein (TP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatine

(CR), total bilirubin (T-BIL), albumin (ALB), albumin globulin ratio (A/G ratio), total cholesterol (TCH), γ-glutamyl transpeptidase (γ-GT), Uric acid (UA) were determined by Microlab-300 autoanalyzer (Merck Pvt. Ltd., Mumbai, India). Serum electrolytes such as sodium (Na) and potassium (K) were measured by EasyLyte Na/K Analyzer (Medica, USA).

2.10. Synovial fluid analysis

2.10.1. Synovial fluid collection

Synovial fluid samples were collected from rats by arthrocentesis in anesthetic condition. To prevent contamination of samples, hair over the talar joint (right hindlimb) was removed and the area was scrubbed using Providone-iodine (Betadine) solution. Then skin over the joint was cleaned; a 25-gauge 0.98-in. sterile needle attached to 6-mL sterile syringe was inserted through the joint capsule and into the space around the joint and synovial fluid was collected into the syringe. Collected fluid sample from each joint was divided into two aliquots, one aliquot was placed in a sterile plain tube for biochemical analysis and the other was placed in a tube containing EDTA-2K (Merck Pvt. Ltd., Mumbai, India) for cytological analysis. Then analysis was done within 1 h of sample collection.

2.10.2. Biochemical analysis of synovial fluid

Synovial fluid samples were centrifuged at 3000 rpm for 10 min within 1 h after collection, and then supernatant was separated. Biochemical parameters including glucose (GLU), total protein (TP), lactate dehydrogenase (LDH), albumin (ALB), globulin (GLB), albumin globulin ratio (A/G ratio), uric acid (UA) were determined by Microlab-300 autoanalyzer (Merck Pvt. Ltd., Mumbai, India).

2.10.3. Cytological examination of synovial fluid

Cell analysis was performed within 1 h after synovial fluid collection. Total nucleated cell count (TNCC) and WBC differential counts (lymphocyte, monocyte, neutrophil, eosinophil) were examined by Medonic CA-620 cell analyzer systems (Boule Medical, Stockholm, Sweden).

2.10.4. Polarized light microscopy examination of synovial fluid

Samples were examined immediately after arthrocentesis by polarized light (Labophot-2, Nikon, with first order red compensator) for the presence of microcrystals. Presence of monosodium urate crystals (MSU) in synovial fluid samples was investigated immediately after extraction of the fluid by simple polarized light microscopy (magnification, 400×); when crystals were detected, their negative bi-refringence was ascertained by using a first-order red compensator (Phelps et al., 1968; Eliseo et al., 1999).

2.11. Phototoxicity test

Phototoxicity tests were performed by the methods described by Marutani et al. (1993), Wagai et al. (1990) and Wagai and Tawara (1991), with some modifications. Redness of ears of rat was used as a phototoxicity index and was scored as follows at 0, 24 and 48 h after the irradiation: 0, normal; 1, mild erythema; 2, moderate erythema; 3, severe erythema and edema formation.

2.12. Gross observation and organ weight

Complete gross postmortem examinations were performed on all terminated and dead animals. All surviving animals were sacrificed by cervical decapitation under anesthesia at the end of the observation period and examine carefully for macroscopic abnormalities. Absolute and relative (organ-to-body weight ratios) weights of major organs and tissues including heart, liver, lungs, spleen, kidney, brain, testes, stomach, adipose tissue and ovaries were measured.

2.13. Histopathology

After cervical decapitation under anesthesia, following samples of major organs and tissues like heart, liver, lung, spleen, kidney, brain, stomach, adipose tissue, together with colon, duodenum, epididymes, pancreas, esophagus, adrenal gland, urinary bladder, uterus, prostate, Achilles tendon and talar joint (right hindlimb) were preserved in neutral, phosphate-buffered 10% formalin. Testes were preserved in Bouin's fixative. Samples were routinely processed, embedded in paraffin, and sectioned at 3–5 μm. Then sections were stained with hematoxylin–eosin stain for microscopic examination. All organs and tissues taken from all animals of the vehicle control and high dose groups were examined microscopically.

2.14. Bioanalytical method

Analyte was separated on a Peerless basic C(18) column (33 mm × 4.6 mm, 3 μm particle size) (Chromatopak, Mumbai, India) with an isocratic mobile phase of methanol and water containing 0.5% (v/v) formic (9:1, v/v) at a flow rate of 0.5 mL/min and was delivered into the mass spectrometer's electrospray ionization chamber. Total run time was 3 min. Analysis was performed in multiple reaction-monitoring (MRM) mode by monitoring the ion transitions from m/z 350.500 → 248.500 (ULF) and m/z 332.400 → 231.400 (IS). Response of ULF was linear over the range 0.010–2.500 μg/mL in all matrixes. Limit of detection (LOD) and lower limit of quantification (LLOQ) of ULF were determined in all matrixes to be 0.0025 μg/mL and 0.010 μg/mL, respectively. Liquid chromatographic (LC) system consists of LC-20AD pump, SIL-20AC autosampler and CTO-10ASvp column oven (Shimadzu, Kyoto, Japan). 30 mL aliquots of processed samples were injected using an SIL 20AC autosampler from Shimadzu (Kyoto, Japan). Quantitation was achieved by MS/MS detection in positive ion modes for both of the analyte and IS using a triple quadrupole mass spectrometer (API 2000) made by AB Sciex Instruments (Foster, CA; Model: 029345-K) equipped with a turboionspray interface at 450 °C. The MS/MS conditions were as follows: ions spray voltage, 5500 V; source temperature, 450 °C; nebulizer gas, 50 psi; auxillary gas, 55 psi; curtain gas, 15 on an arbitrary scale; collision gas, 7 on an arbitrary scale. Quadrupoles Q1 and Q3 were set at unit resolution. Instrument was controlled and data integration was performed with analyst 1.5 software version (AB Sciex Instruments, Foster, CA).

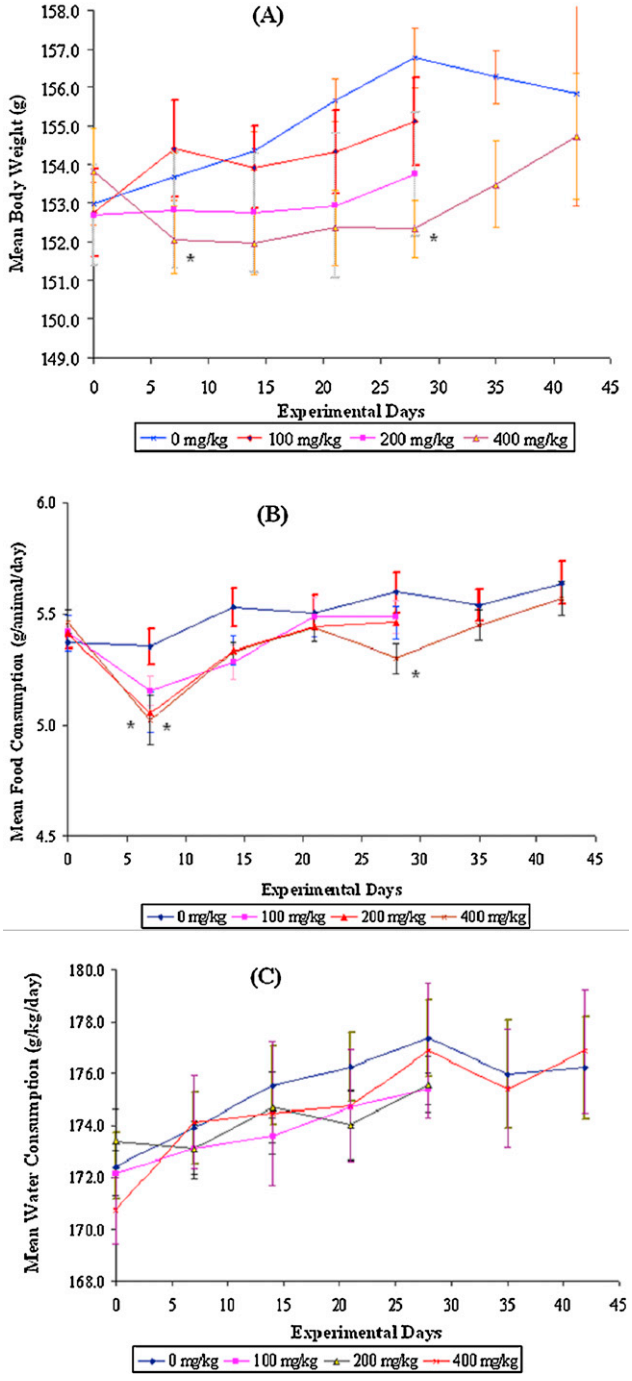

Fig. 2 – Mean body weight (A), mean food consumption (B), and mean water consumption (C) trends for male Wister albino rats dosed once with prulifloxacin at 100, 200, or 400 mg/kg/body weight. Each data point represents the mean ± SEM ($n = 12$). *Significantly different from control groups ($P \leq 0.05$). Standard error of the mean (SEM) = standard deviation (S.D.)/√total subject.

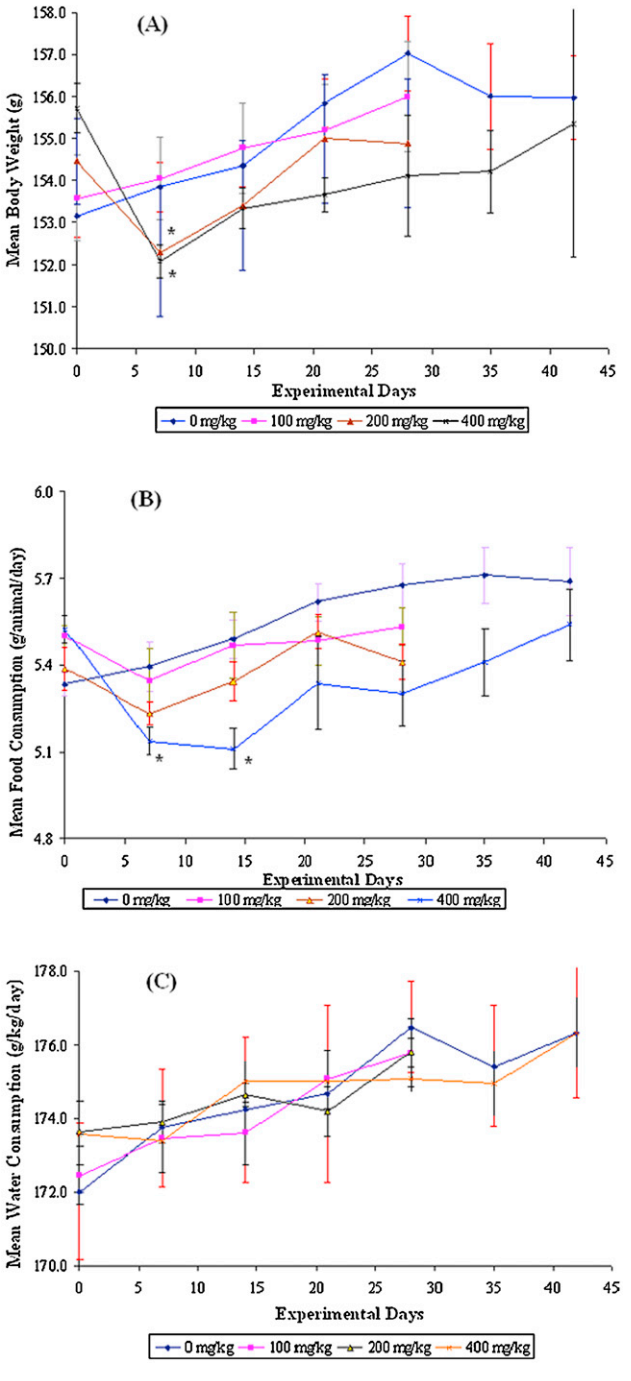

Fig. 3 – Mean body weight (A), mean food consumption (B), and mean water consumption (C) trends for female Wister albino rats dosed once with prulifloxacin at 100, 200, or 400 mg/kg/body weight. Each data point represents the mean ± SEM ($n = 12$). *Significantly different from control groups ($P \leq 0.05$). Standard error of the mean (SEM) = standard deviation (S.D.)/√total subject.

Table 1 – Urine analysis in male and female rats of rats treated with prulifloxacin after treatment (28-days) and recovery (14-days) period.

Parameters (mean ± SEM)	After treatment period (28th day) at the doses (mg/kg/day) ($n = 12$ in each group)				After recovery period (42nd day) at the doses (mg/kg/day) ($n = 6$ in each group)	
	0	100	200	400	0	400
Male						
Ketone (mg/100 mL)	10.18 ± 0.62	9.84 ± 0.94	11.33 ± 0.84	11.78 ± 1.08	10.27 ± 0.98	10.962 ± 1.02
Protein (mg/100 mL)	20.22 ± 2.95	33.41 ± 3.10	42.30 ± 2.74	69.10 ± 3.21**	24.10 ± 3.61	31.44 ± 2.81
pH	7.22 ± 0.18	6.74 ± 0.28	6.87 ± 0.23	6.18 ± 0.14*	6.64 ± 0.21	6.80 ± 0.15
Leucocytes (WBC/μL)	23.28 ± 3.61	28.66 ± 1.98	38.95 ± 3.22	79.41 ± 3.86*	29.27 ± 1.84	30.22 ± 2.08
Specific gravity (g/mL)	1.114 ± 0.04	1.094 ± 0.05	1.101 ± 0.07	1.129 ± 0.06	1.028 ± 0.03	1.015 ± 0.06
Glucose (mg/dL)	55.10 ± 2.19	59.32 ± 2.91	61.98 ± 3.22	78.10 ± 3.74*	59.37 ± 2.21	60.19 ± 4.01
Creatinine (mg/dL)	95.11 ± 2.10	88.30 ± 1.68	82.67 ± 3.15	61.44 ± 2.30**	89.97 ± 3.20	88.22 ± 1.91
Na^+ (mEq/day)	1.24 ± 0.34	1.10 ± 0.44	0.97 ± 0.24	0.58 ± 0.62**	1.18 ± 0.30	1.01 ± 0.71
K^+ (mEq/day)	2.80 ± 0.51	2.81 ± 0.24	2.74 ± 0.33	2.24 ± 0.22*	2.75 ± 0.44	2.69 ± 0.34
Volume (mL/day)	8.22 ± 1.89	7.98 ± 2.42	9.30 ± 3.01	12.46 ± 4.15**	8.61 ± 2.08	8.83 ± 3.04
Female						
Ketone (mg/100 mL)	9.85 ± 0.55	10.19 ± 0.39	11.24 ± 1.42	11.09 ± 0.74	10.38 ± 1.04	10.76 ± 0.88
Protein (mg/100 mL)	21.30 ± 3.29	24.98 ± 1.08	42.18 ± 2.45	78.36 ± 3.10**	29.31 ± 3.16	30.98 ± 1.52
pH	7.09 ± 0.11	7.10 ± 0.21	6.88 ± 0.51	6.20 ± 0.17**	6.98 ± 0.20	6.82 ± 0.18
Leucocytes (WBC/μL)	20.27 ± 3.11	35.41 ± 2.29	39.15 ± 2.65	82.33 ± 4.10*	26.44 ± 2.30	33.32 ± 3.20
Specific gravity (g/mL)	1.055 ± 0.05	1.021 ± 0.01	1.121 ± 0.02*	1.122 ± 0.04*	1.014 ± 0.01	1.022 ± 0.02
Glucose (mg/dL)	57.22 ± 1.88	60.22 ± 3.24	65.34 ± 2.20	81.25 ± 1.88**	60.34 ± 1.95	61.33 ± 2.07
Creatinine (mg/dL)	96.32 ± 2.54	87.65 ± 2.10	88.30 ± 2.65	65.44 ± 3.20*	91.30 ± 1.95	89.30 ± 2.30
Na^+ (mEq/day)	1.18 ± 0.29	1.22 ± 0.50	0.96 ± 0.30	0.60 ± 0.28*	1.20 ± 0.40	1.12 ± 0.34
K^+ (mEq/day)	2.84 ± 0.61	2.78 ± 0.42	2.64 ± 0.25	2.20 ± 0.14**	2.82 ± 0.50	2.71 ± 0.36
Volume (mL/day)	8.64 ± 1.08	8.09 ± 3.22	9.21 ± 1.69	12.89 ± 2.01**	8.58 ± 3.26	8.77 ± 2.30

* Significant difference at $P < 0.05$ level, when compared with the control group.
** Significant difference at $P < 0.01$ level, when compared with the control group.

2.15. *Toxicokinetics and tissue distribution study*

Blood samples were collected into the tubes containing EDTA-2K (Merck Pvt. Ltd., Mumbai, India) at a predetermined time interval of 0.5, 1.5, 2, 4, 6, 8, 12, and 24 h after administration of PRF on day-1 and -28. Plasma was separated by centrifugation of blood and stored at −20 °C until analysis. Tissue samples were collected at the end of treatment (28th day) and recovery period (42nd day).

2.15.1. *Plasma samples preparation*

Samples were prepared by taking 225 μL of plasma sample, adding IS solution (25 μL of 1 μg/mL; equivalent to 0.025 μg) and vortex mixing for 1 min, respectively. Then 100 μL of 10 mM tris buffer [tris (hydroxymethyl) aminomethane] was added and vortex mixing for 30 s. Volume make up to 2.500 mL was done by extracting solvent of a mixture of chloroform–isoamyl alcohol (85:15, v/v) and contents were hand mixed for 10 min, followed by centrifugation at 5000 rpm for 15 min. Organic layer (2.250 mL) was separated and evaporated to dryness at 40 °C using a gentle stream of nitrogen. Residue was reconstituted in 125 μL of mobile phase containing 10% tris buffer (10 mM) and 30 μL was injected onto the LC–MS/MS system.

2.15.2. *Tissue samples preparation*

Following method of sample preparation was used for all tissues including liver, lung, kidney, brain, heart, testes, stomach and adipose tissue of Wistar albino rats. Tissues (0.3 g) were homogenized twice in 1 mL homogenate media [phosphate buffered saline (PBS) consists of 0.2 M potassium phosphate, 1.5 M sodium chloride, pH 7.2, prepared in Molecular Biology Grade water (DEPC treated)] for 30 s per time using an Ultra-Turrax T25 tissue disperser (IKA-Labortechnik, Germany) in an ice bath. Homogenized samples were sonicated for 5 min and centrifuged at 3500 rpm for 10 min. Resultant supernatants were stored in polypropylene tube individually for each tissue. These supernatants were represents as like as liquid plasma and were used for bio-analytical sample preparation. At first, we validated the developed LC–MS/MS method in different tissues of Wistar albino rats and then proceed to quantify ULF in these tissues. Tissue samples were prepared through same method which has been used for plasma samples preparation. Plasma concentration–time data of each analyte was estimated by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA) software.

On day-28, tissue distribution of ULF (concentration in different tissue) was measured with the help of calibration curve prepared for each tissue. Tissue accumulation of ULF was measured on day-42 in remaining rats of high dose group with the help of calibration curve.

2.16. *Statistical analysis*

Statistical analysis was performed by comparing treatment groups with vehicle control group. Variance of numerical data was checked by Bartlett's test. Levene's test was performed to determine whether the groups had equal variances. If the

Table 2 – Hematological findings in male and female rats treated with prulifloxacin for treatment (28-days) and recovery (14 days) period.

Parameters (mean ± SEM)	After treatment period (day 28) at the doses (mg/kg/day) ($n = 12$ in each group)				After recovery period (day 42) at the doses (mg/kg/day) ($n = 6$ in each group)	
	0	100	200	400	0	400
Male						
Hemoglobin (g %)	13.89 ± 0.31	14.01 ± 0.22	13.15 ± 0.22	14.26 ± 0.30	14.32 ± 0.44	14.11 ± 0.44
Total RBC ($\times 10^6$/cmm)	6.22 ± 0.20	6.50 ± 0.41	6.32 ± 0.30	6.40 ± 0.26	6.26 ± 0.24	6.28 ± 0.20
Reticulocytes (%)	1.36 ± 0.22	1.41 ± 0.14	1.54 ± 0.20	1.40 ± 0.17	1.45 ± 0.30	1.40 ± 0.16
Hematocrit (%)	43.49 ± 0.54	42.40 ± 1.08	41.82 ± 1.11	44.24 ± 1.08	42.31 ± 0.90	42.65 ± 0.74
MCV (μm^3)	69.32 ± 3.48	66.74 ± 3.56	70.24 ± 4.08	68.55 ± 4.22	68.22 ± 3.88	68.31 ± 4.19
MCH (pg)	21.41 ± 1.14	22.44 ± 1.17	23.110 ± 1.20	23.41 ± 1.19	23.10 ± 1.65	22.89 ± 1.41
MCHC (%)	32.04 ± 0.25	33.41 ± 0.22	34.10 ± 0.42	34.01 ± 0.31	32.28 ± 0.32	33.04 ± 0.30
Platelets ($\times 10^5$/cmm)	3.09 ± 0.22	3.28 ± 0.20	3.41 ± 0.36	4.42 ± 0.30**	3.13 ± 0.28	3.48 ± 0.45
Total WBC ($\times 10^3$/cmm)	8.41 ± 0.34	9.18 ± 0.51	8.84 ± 0.63	12.64 ± 0.32*	8.80 ± 0.48	8.82 ± 0.50
Neutrophils (%)	20.41 ± 0.56	22.33 ± 0.62	21.08 ± 0.72	20.84 ± 0.45	19.57 ± 0.84	21.08 ± 0.63
Lymphocytes (%)	75.41 ± 0.90	76.70 ± 0.84	76.08 ± 0.58	77.24 ± 0.40	76.41 ± 0.85	76.88 ± 0.38
Eosinophils (%)	1.95 ± 0.30	2.14 ± 0.34	2.02 ± 0.20	1.81 ± 0.29	2.401 ± 0.34	1.99 ± 0.25
Monocytes (%)	0.54 ± 0.14	0.48 ± 0.20	0.56 ± 0.15	0.51 ± 0.18	0.49 ± 0.21	0.55 ± 0.20
Basophils (%)	0.00 ± 0.00	0.08 ± 0.08	0.17 ± 0.23	0.08 ± 0.04	0.00 ± 0.00	0.25 ± 0.13
Female						
Hemoglobin (g %)	14.11 ± 0.40	13.84 ± 0.41	14.14 ± 0.28	14.57 ± 0.55	14.20 ± 0.54	14.35 ± 0.28
Total RBC ($\times 10^6$/cmm)	6.78 ± 0.41	7.02 ± 0.35	6.92 ± 0.30	7.22 ± 0.50	6.72 ± 0.44	7.16 ± 0.35
Reticulocytes (%)	1.41 ± 0.15	1.39 ± 0.14	1.42 ± 0.20	1.40 ± 0.14	1.43 ± 0.09	1.41 ± 0.19
Hematocrit (%)	42.54 ± 1.09	43.22 ± 1.62	44.25 ± 1.19	44.22 ± 1.34	43.05 ± 1.42	42.34 ± 1.74
MCV (μm^3)	67.22 ± 2.33	64.52 ± 3.56	61.86 ± 3.02	64.85 ± 4.08	66.75 ± 3.55	65.08 ± 2.69
MCH (pg)	21.33 ± 0.65	20.45 ± 1.09	22.71 ± 1.36	21.18 ± 1.25	22.20 ± 1.17	20.58 ± 1.43
MCHC (%)	32.40 ± 0.25	33.14 ± 0.18	34.14 ± 0.25	33.40 ± 0.49	34.22 ± 0.29	32.95 ± 0.44
Platelets ($\times 10^5$/cmm)	3.20 ± 0.30	3.31 ± 0.22	3.68 ± 0.40	3.65 ± 0.34	3.52 ± 0.24	3.48 ± 0.40
Total WBC ($\times 10^3$/cmm)	7.89 ± 0.33	8.22 ± 0.41	8.68 ± 0.41	12.51 ± 0.19**	8.22 ± 0.34	8.49 ± 0.42
Neutrophils (%)	22.24 ± 0.49	20.35 ± 0.42	21.19 ± 0.72	21.25 ± 0.68	21.05 ± 1.05	22.84 ± 0.68
Lymphocytes (%)	74.89 ± 0.86	76.99 ± 0.81	77.01 ± 0.75	78.25 ± 0.96	74.84 ± 1.01	77.02 ± 0.44
Eosinophils (%)	1.85 ± 0.19	1.92 ± 0.20	1.95 ± 0.20	2.13 ± 0.25	2.19 ± 0.15	2.02 ± 0.20
Monocytes (%)	0.62 ± 0.10	0.57 ± 0.20	0.59 ± 0.20	0.65 ± 0.15	0.58 ± 0.16	0.61 ± 0.09
Basophils (%)	0.08 ± 0.08	0.08 ± 0.08	0.00 ± 0.00	0.04 ± 0.11	0.15 ± 0.07	0.08 ± 0.08

MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RBC: red blood cell; WBC: white blood cell.

* Significant difference at $P < 0.05$ level, when compared with the control group.

** Significant difference at $P < 0.01$ level, when compared with the control group.

variance was homogeneous, then data was subjected to one-way analysis of variance (ANOVA). If not, they were analyzed by the Kruskal–Wallis non-parametric ANOVA. If either of the tests showed a significant difference among the groups, the data were analyzed by the multiple comparison procedure of the Dunnett's *post hoc* test. Histopathological findings were represented as frequencies and were subjected to the Fisher's exact probability test when necessary. Data were presented as mean ± SEM. All tests were two-tailed and the level of significance was taken as $P < 0.05$ or 0.01.

To determine the toxicokinetic parameters of PRF, plasma concentration–time data were analyzed by non-compartmental analysis method. Area under the plasma concentration–time curve (AUC) from 0 to 24 h after administration was calculated by the linear trapezoidal method. Mean plasma concentration–time profiles of PRF per dose group were obtained by averaging the individual concentrations per sampling time point and by plotting the mean concentrations *versus* sampling time point. C_{max} was defined as the highest observed concentration of PRF in plasma and t_{max} was defined as the time to reach C_{max}. Half-lives were determined from rate constants using the relationship: half life = ln 2/*k*. Relationship between dose and exposure was assessed by plotting the $AUC_{0-24\,h}$ or C_{max} values *versus* oral dose. All results were expressed as arithmetic mean ± standard deviation (S.D.). Comparisons between the groups were analyzed using the Student's *t*-test (two-tailed). Differences were considered to be statistically significant when *P*-value were less than 0.05. The calculations were performed in Microsoft Excel (V. 2003).

3. Results

3.1. *Clinical sign and mortality*

Rats of 400 mg/kg/day dose group showed making noise immediately after treatment (data not shown). These phenomenons were also present to a lesser extent in the 200 mg/kg/day dose group. There was no treatment-related mortality in animals treated with PRF at 100, 200, and 400 mg/kg/day for 28 days.

Table 3 – Serum biochemical findings in male rats treated with prulifloxacin for treatment (28-days) and recovery (14-days) period.

Parameters (mean ± SEM)	After treatment period (day 28) at the doses (mg/kg/day) ($n = 12$ in each group)				After recovery period (day 42) at the doses (mg/kg/day) ($n = 6$ in each group)	
	0	100	200	400	0	400
Male						
Aspartate aminotransferase (IU/L)	91.40 ± 3.16	88.10 ± 2.64	90.40 ± 4.05	92.05 ± 3.24	90.05 ± 2.54	90.05 ± 3.22
Alanine aminotransferase (IU/L)	40.22 ± 1.32	42.04 ± 0.88	45.35 ± 0.48	61.25 ± 1.44**	42.35 ± 1.05	44.35 ± 1.74
Alkaline phosphatase (IU/L)	164.33 ± 3.85	166.24 ± 3.24	168.77 ± 4.65	165.33 ± 4.04	163.10 ± 4.55	161.34 ± 5.30
Blood urea nitrogen (mg/dL)	15.11 ± 0.55	14.85 ± 0.52	15.87 ± 0.81	14.66 ± 0.68	14.95 ± 0.75	15.01 ± 0.64
Creatinine (mg/dL)	0.56 ± 0.03	0.55 ± 0.01	0.58 ± 0.02	0.56 ± 0.02	0.57 ± 0.01	0.58 ± 0.01
Glucose (mg/dL)	93.65 ± 1.75	96.44 ± 1.44	95.05 ± 1.25	114.64 ± 1.44**	95.38 ± 1.20	95.32 ± 1.64
Total cholesterol (mg/dL)	97.44 ± 0.65	98.26 ± 0.44	101.02 ± 0.44	98.74 ± 1.08	98.01 ± 1.08	99.32 ± 0.94
Total bilirubin (mg/dL)	0.05 ± 0.005	0.06 ± 0.001	0.08 ± 0.008*	0.09 ± 0.010**	0.06 ± 0.006	0.06 ± 0.005
Total protein (g/dL)	6.88 ± 0.42	6.48 ± 0.30	6.59 ± 0.32	5.091 ± 0.24*	6.79 ± 0.36	6.485 ± 0.30
Triglyceride (mg/dL)	141.25 ± 3.56	144.29 ± 2.38	142.85 ± 4.56	144.35 ± 5.24	142.44 ± 4.32	140.32 ± 5.41
Albumin (g/dL)	4.22 ± 0.10	4.32 ± 0.12	4.09 ± 0.11	4.40 ± 0.18	4.24 ± 0.18	4.21 ± 0.18
Inorganic phosphate (mg/dL)	4.58 ± 0.15	4.62 ± 0.11	4.76 ± 0.18	4.84 ± 0.21	4.63 ± 0.20	4.64 ± 0.21
Phospholipid (mg/dL)	160.11 ± 3.52	158.25 ± 4.05	155.63 ± 3.10	154.46 ± 3.15	158.50 ± 4.45	155.25 ± 5.44
Albumin/globulin (ratio)	1.72 ± 0.09	1.79 ± 0.08	1.89 ± 0.09	2.04 ± 0.08	1.68 ± 0.11	1.75 ± 0.10
Uric acid (μmol/L)	442.24 ± 3.48	451.34 ± 2.08	450.60 ± 3.44	444.60 ± 4.20	451.47 ± 2.05	447.30 ± 3.84
Sodium (nmol/L)	140.78 ± 1.35	140.55 ± 1.04	141.20 ± 1.24	144.24 ± 0.88	141.371 ± 1.48	142.62 ± 1.44
Potassium (nmol/L)	4.85 ± 0.10	5.01 ± 0.12	4.95 ± 0.09	5.08 ± 0.14	5.05 ± 0.10	4.98 ± 0.11

Standard error of the mean (SEM) = standard deviation (S.D.)/√total subject.
* Significant difference at $P < 0.05$ level.
** Significant difference at $P < 0.01$ level.

3.2. *Consumption and body weight trends*

Food consumption temporarily was suppressed in the 400 mg/kg/day dose group of both sex but returned toward normal levels during recovery period. In male rats, food consumption was significantly decreased on day-7 and day-28 in the high dose group than in the vehicle control group. In female rats, food consumption was also significantly

Table 4 – Serum biochemical findings in female rats treated with prulifloxacin for treatment (28-days) and recovery (14-days) period.

Parameters (mean ± SEM)	After treatment period (day 28) at the doses (mg/kg/day) ($n = 12$ in each group)				After recovery period (day 42) at the doses (mg/kg/day) ($n = 6$ in each group)	
	0	100	200	400	0	400
Female						
Aspartate aminotransferase (IU/L)	96.24 ± 2.04	94.78 ± 1.10	95.87 ± 2.15	96.48 ± 1.45	95.45 ± 3.10	95.88 ± 2.40
Alanine aminotransferase (IU/L)	42.24 ± 0.98	44.19 ± 0.88	48.97 ± 0.98*	64.22 ± 1.44**	44.02 ± 1.34	45.01 ± 2.01
Alkaline phosphatase (IU/L)	159.01 ± 2.48	158.25 ± 3.05	161.24 ± 2.44	160.20 ± 3.44	158.06 ± 3.42	158.44 ± 4.22
Blood urea nitrogen (mg/dL)	14.68 ± 0.88	15.08 ± 1.06	15.24 ± 0.54	14.68 ± 0.32	5.20 ± 0.64	15.05 ± 1.08
Creatinine (mg/dL)	0.60 ± 0.08	0.58 ± 0.04	0.60 ± 0.02	0.59 ± 0.05	0.59 ± 0.09	0.58 ± 0.07
Glucose (mg/dL)	95.24 ± 1.08	96.08 ± 1.14	98.14 ± 1.24	113.14 ± 0.88**	96.95 ± 1.20	97.84 ± 0.98
Total cholesterol (mg/dL)	98.10 ± 0.85	99.21 ± 0.90	99.22 ± 0.35	98.88 ± 0.44	99.10 ± 1.04	99.24 ± 0.92
Total bilirubin (mg/dL)	0.06 ± 0.008	0.06 ± 0.004	0.07 ± 0.001	0.10 ± 0.008**	0.06 ± 0.001	0.07 ± 0.005
Total protein (g/dL)	6.98 ± 0.21	6.74 ± 0.15	6.24 ± 0.20	5.08 ± 0.19*	6.90 ± 0.40	6.48 ± 0.37
Triglyceride (mg/dL)	144.40 ± 4.52	145.34 ± 3.45	140.34 ± 4.21	142.42 ± 4.65	140.32 ± 6.22	139.41 ± 4.81
Albumin (g/dL)	4.35 ± 0.09	4.24 ± 0.10	4.22 ± 0.09	4.38 ± 0.20	4.55 ± 0.20	4.32 ± 0.30
Inorganic phosphate (mg/dL)	4.64 ± 0.15	4.57 ± 0.10	4.49 ± 0.14	4.66 ± 0.10	4.44 ± 0.10	4.51 ± 0.18
Phospholipid (mg/dL)	161.34 ± 2.55	160.20 ± 4.22	158.50 ± 2.85	161.22 ± 4.10	160.47 ± 4.02	162.34 ± 4.10
Albumin/globulin (ratio)	1.94 ± 0.11	2.01 ± 0.08	1.98 ± 0.09	2.11 ± 0.09	2.04 ± 0.15	1.96 ± 0.21
Uric acid (μmol/L)	414.32 ± 2.45	410.41 ± 2.21	405.82 ± 3.05	418 ± 2.42	422.27 ± 4.12	414.28 ± 5.10
Sodium (nmol/L)	140.24 ± 1.11	144.24 ± 0.98	142.44 ± 2.04	141.44 ± 3.35	140.65 ± 1.35	141.87 ± 2.09
Potassium (nmol/L)	5.04 ± 0.14	4.58 ± 0.08	5.11 ± 0.11	4.98 ± 0.07	5.18 ± 0.18	5.15 ± 0.16

Standard error of the mean (SEM) = standard deviation (S.D.)/√total subject.
* Significant difference at $P < 0.05$ level, when compared with the control group.
** Significant difference at $P < 0.01$ level, when compared with the control group.

Table 5 – Biochemical analysis of synovial fluid of rats treated with prulifloxacin for treatment (28-days) and recovery (14-days) period.

Parameters (mean ± SEM)	After treatment period (day 28) at the doses (mg/kg/day) ($n = 12$ in each group)				After recovery period (day 42) at the doses (mg/kg/day) ($n = 6$ in each group)	
	0	100	200	400	0	400
Male						
Glucose (mg/dL)	48.14 ± 0.35	44.15 ± 0.28	41.62 ± 0.40	36.87 ± 0.38**	46.58 ± 0.67	46.07 ± 0.51
Total protein (g/dL)	2.09 ± 0.05	2.34 ± 0.03	2.64 ± 0.02	3.49 ± 0.05*	2.39 ± 0.06	2.61 ± 0.04
Albumin/globulin (ratio)	2.55 ± 0.09	2.38 ± 0.07	2.82 ± 0.19	2.66 ± 0.08	2.58 ± 0.12	2.54 ± 0.08
Albumin (g/dL)	1.48 ± 0.02	1.45 ± 0.07	1.42 ± 0.01	1.49 ± 0.02	1.47 ± 0.03	1.48 ± 0.28
Globulin (g/dL)	0.82 ± 0.02	0.78 ± 0.01	0.80 ± 0.06	0.81 ± 0.03	0.81 ± 0.04	0.79 ± 0.02
Uric acid (μmol/L)	394.34 ± 3.45	384.32 ± 3.42	408.45 ± 4.22	496.14 ± 2.98**	408.42 ± 4.35	415.21 ± 4.25
Lactate dehydrogenase (IU/L)	286.24 ± 2.84	301.52 ± 3.19	299.56 ± 4.20	361.65 ± 3.41*	314.33 ± 4.10	322.65 ± 3.48
Female						
Glucose (mg/dL)	49.14 ± 0.34	45.96 ± 0.42	43.65 ± 0.55	36.841 ± 0.41**	48.46 ± 0.44	45.33 ± 0.44
Total protein (g/dL)	2.11 ± 0.04	2.34 ± 0.05	2.43 ± 0.01	3.42 ± 0.05**	2.38 ± 0.01	2.35 ± 0.06
Albumin/globulin (ratio)	2.74 ± 0.32	2.68 ± 0.16	2.54 ± 0.13	2.75 ± 0.09	2.69 ± 0.06	2.72 ± 0.32
Albumin (g/dL)	1.51 ± 0.04	1.48 ± 0.05	1.50 ± 0.08	1.49 ± 0.05	1.50 ± 0.06	1.51 ± 0.09
Globulin (g/dL)	0.80 ± 0.06	0.82 ± 0.03	0.78 ± 0.01	0.83 ± 0.04	0.79 ± 0.02	0.80 ± 0.02
Uric acid (μmol/L)	403.25 ± 3.12	408.34 ± 4.12	412.40 ± 3.68	497.41 ± 3.29**	410.30 ± 4.62	424.26 ± 5.10
Lactate dehydrogenase (IU/L)	298.65 ± 3.11	306.22 ± 2.95	325.44 ± 2.33	354.10 ± 4.11*	312.05 ± 4.22	319.26 ± 4.49

Standard error of the mean (SEM) = standard deviation (S.D.)/√total subject.
* Significant difference at $P < 0.05$ level, when compared with the control group.
** Significant difference at $P < 0.01$ level, when compared with the control group.

decreased on day-7 and day-14 in the high dose group. Mean body weight of animals in the high dose group was significantly lower than vehicle control group throughout the exposure period. Water consumption of treated and control groups were similar for each sex. Mean water and food consumption trends and the effects of PRF in body weight are illustrated in Figs. 2 and 3 for male and female rats, respectively.

3.3. *Urinalysis*

In urinalysis, crystalline substance in the urinary sediments, cloudy urine, decreased Na(+) excretion were observed in the 200 and 400 mg/kg/day dose groups. A similar increased of urine volume, urinary proteins, glucose, creatinine and leucocytes count, and decreased of urinary pH, urine specific gravity and decreased K(+) and Cl(−) excretions were observed in high dose group of both sex during treatment period of repeated oral toxicity study but were returned toward normal range after recovery period (Table 1).

3.4. *Hematology*

On day-28, significant increase of WBC count was seen for males and females and platelet counts in males treated with 400 mg/kg/day dose group (Table 2) when compared with

Table 6 – Cytologic examination of synovial fluid in male and female rats treated with prulifloxacin for treatment (28-days) and recovery (14 days) period.

Parameters (mean ± SEM)	After treatment period (day 28) at the doses (mg/kg/day) ($n = 12$ in each group)				After recovery period (day 42) at the doses (mg/kg/day) ($n = 6$ in each group)	
	0	100	200	400	0	400
Male						
TNCC (Cell/μL)	184.52 ± 3.24	191.44 ± 3.24	197.54 ± 3.21	258.36 ± 3.22**	192.15 ± 4.12	198.33 ± 4.41
Neutrophils (%)	2.14 ± 0.06	2.38 ± 0.05	2.68 ± 0.09	3.41 ± 0.04**	2.41 ± 0.08	2.54 ± 0.11
Lymphocytes (%)	46.18 ± 0.30	48.14 ± 0.33	47.75 ± 0.28	48.08 ± 0.44	45.71 ± 0.39	47.10 ± 0.23
Monocytes (%)	42.33 ± 0.19	44.31 ± 0.10	39.45 ± 0.41	41.77 ± 0.42	39.84 ± 0.25	40.84 ± 0.26
Female						
TNCC (Cell/μL)	190.22 ± 2.45	193.12 ± 3.22	198.54 ± 3.65	254.32 ± 3.42*	189.57 ± 4.02	201.22 ± 2.11
Neutrophils (%)	2.48 ± 0.08	2.44 ± 0.09	2.40 ± 0.05	3.49 ± 0.07*	2.60 ± 0.01	2.74 ± 0.02
Lymphocytes (%)	47.20 ± 0.11	46.18 ± 0.14	45.37 ± 0.15	48.15 ± 0.40	48.26 ± 0.34	49.45 ± 0.20
Monocytes (%)	40.77 ± 0.08	41.12 ± 0.27	43.22 ± 0.30	41.65 ± 0.37	39.74 ± 0.19	40.94 ± 0.25

Standard error of the mean (SEM) = standard deviation (S.D.)/√total subject.
* Significant difference at $P < 0.05$ level, when compared with the control group.
** Significant difference at $P < 0.01$ level, when compared with the control group.

Table 7 – Phototoxic examination of rats treated with prulifloxacin for treatment (28-days) period.

Compound and dose (mg/kg/day)	No. of animals with the indicated score[a] at:																							
	Treatment period ($n = 12$ in each group)																							
	7th day												14th day											
	0 h				24 h				48 h				0 h				24 h				48 h			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Treatment period																								
Male																								
Vehicle	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0
Prulifloxacin																								
100	0	12	0	0	0	8	3	1	0	1	7	4	0	8	4	0	0	2	9	1	0	5	5	2
200	1	8	3	0	0	7	4	1	0	1	10	1	1	7	4	0	0	2	10	0	0	8	1	3
400	0	1	6	5	0	6	4	2	0	1	2	9	0	1	8	3	0	0	5	7	0	2	1	9
Female																								
Vehicle	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0
Prulifloxacin																								
100	0	10	2	0	0	6	5	1	0	1	9	2	1	9	2	0	0	1	10	1	0	1	7	4
200	0	0	12	0	0	3	7	2	0	1	8	3	0	4	8	0	0	3	9	0	1	0	8	3
400	0	1	10	1	0	6	1	5	0	1	1	10	0	5	5	2	0	0	6	6	0	0	3	9

Compound and dose (mg/kg/day)	No. of animals with the indicated score[a] at:																							
	Treatment period ($n = 12$ in each group)																							
	21st day												28th day											
	0 h				24 h				48 h				0 h				24 h				48 h			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Treatment period																								
Male																								
Vehicle	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0
Prulifloxacin																								
100	1	3	8	0	1	4	7	0	0	2	5	5	0	4	8	0	0	1	5	6	0	2	2	8
200	0	4	7	1	0	4	5	3	0	1	4	7	1	8	3	0	0	1	5	6	0	3	1	8
400	0	4	6	2	0	1	1	1	0	0	3	1	8	0	2	8	2	1	12	8	0	0	0	12
Female																								
Vehicle	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0	12	0	0	0
Prulifloxacin																								
100	0	6	3	3	0	4	6	2	0	0	6	6	0	3	8	1	0	1	3	8	0	0	5	7
200	0	1	9	2	0	2	2	8	0	0	4	8	0	8	3	1	0	0	1	11	0	1	1	10
400	0	2	8	2	0	1	3	8	0	2	1	9	0	2	8	2	0	1	1	10	0	0	0	12

[a] The scores for ear redness were as follows: 0, normal; 1, mild erythema; 2, moderate erythema; 3, severe erythema and edema.

vehicle control group ($P < 0.01$ and $P < 0.05$). At the end of the recovery period, total WBC and platelet counts were returned toward normal range in this group (Table 2).

3.5. *Serum biochemistry*

At the end of the treatment period, significant increases in T-BIL, glucose and ALT in both sexes was found in the 400 mg/kg/day dose group ($P < 0.05$) (Tables 3 and 4). A significant decrease in TP was observed in both sex, treated with the 400 mg/kg/day of PRF ($P < 0.05$) (Tables 3 and 4). At the end of recovery period, no significant differences were noted for any of these serum biochemical parameters between the vehicle control and high dose groups (Tables 3 and 4).

3.6. *Synovial fluid analysis*

3.6.1. *Biochemical analysis of synovial fluid*

After 28-days treatment period, significant increases in LDH, UA and TP in both sex of 400 mg/kg/day dose group were found ($P < 0.01$; $P < 0.05$) (Table 5). A significant decrease in glucose concentration was observed in both sex in high dose level ($P < 0.01$) (Table 5). At the end of recovery period, no significant differences were noted for any of these serum biochemical parameters between vehicle control and high dose groups.

3.6.2. *Cytological examination of synovial fluid*

As shown in Table 6, TNCC and neutrophils in male rats were significantly increased ($P < 0.05$; $P < 0.01$) in the 400 mg/kg/day group when compared with vehicle control

Table 8 – Phototoxic examination of rats treated with prulifloxacin after recovery (14 days) period.

Compound and dose (mg/kg/day)	No. of animals with the indicated score[a] at: After recovery period (42nd day) ($n = 6$ in each group)											
	0 h				24 h				48 h			
	0	1	2	3	0	1	2	3	0	1	2	3
Male												
Vehicle	6	0	0	0	6	0	0	0	6	0	0	0
Prulifloxacin												
400	3	2	1	0	5	1	0	0	4	0	2	0
Female												
Vehicle	6	0	0	0	6	0	0	0	6	0	0	0
Prulifloxacin												
400	4	2	0	0	5	0	1	0	3	3	0	0

[a] The scores for ear redness were as follows: 0, normal; 1, mild erythema; 2, moderate erythema; 3, severe erythema and edema.

group. No eosinophils were observed on cytological examination. At the end of the recovery period, TNCC and neutrophils were returned toward normal range in high dose group (Table 6).

3.6.3. *Polarized light microscopy examination of synovial fluid*

Ninety samples were examined after 28-days treatment period including 24 samples from high dose group. MSU crystals were found in seven samples (4 male and 3 female) of middle dose (200 mg/kg/day) and twenty one samples (10 male and 11 female) of high dose group (400 mg/kg/day). CPPD crystals were found in six samples (2 male and 4 female) of middle dose group (200 mg/kg/day) and nineteen samples (10 male and 9 female) of high dose group (400 mg/kg/day). No crystals were seen in vehicle control group and low dose level group (100 mg/kg/day). After recovery period MSU crystal in two samples (3 male) and CPPD crystal in five samples (3 male and 2 female) of high dose group (400 mg/kg/day) were found.

3.7. *Phototoxicity test*

As shown in Tables 7 and 8, PRF produces ear erythema and redness of the ears after 7 days treatment in rats of both sex in 400 mg/kg/day dose group. In recovery period this phototoxic effect (ear erythema) was not appeared.

3.8. *Gross observation and organ weight*

Some animals of both sex in the high dose group were also observed to have joint swelling of the hindlimb. At necropsy, nine animals of 400 mg/kg/day dose group showed cyst, filled with yellowish materials and congestion of auxiliary area, rupture of the esophagus, dark red discoloration on the adrenal gland. Absolute spleen weight and relative salivary gland weight were also decreased, while relative liver weight was significantly increased in both sex of 400 mg/kg/day dose group. Liver:body weight ratio was significantly increased in both sex at high dose level. Terminal body weights, absolute and relative liver weights and liver: body weight ratio of rats

Table 9 – Terminal body weights, absolute liver weights and relative liver weights of rats treated with prulifloxacin after treatment (28 days) and recovery (14-days) period.

Parameters (mean ± SEM)	After treatment period (28th day) at the doses (mg/kg/day) ($n = 12$ in each group)				After recovery period (42nd day) at the doses (mg/kg/day) ($n = 6$ in each group)	
	0	100	200	400	0	400
Male						
Terminal body weight (g)	156.76 ± 0.78	155.12 ± 1.14	153.75 ± 1.60	152.34 ± 0.75	155.84 ± 4.09	154.74 ± 1.62
Absolute liver weight (g)	5.21 ± 0.09	5.40 ± 0.07	5.67 ± 0.10*	5.80 ± 0.11*	5.42 ± 0.13	5.45 ± 0.08
Relative liver weight	3.38 ± 0.05	3.57 ± 0.05	3.72 ± 0.06*	3.94 ± 0.09**	3.49 ± 0.13	3.52 ± 0.06
Female						
Terminal body weight (g)	157.03 ± 0.88	156.00 ± 1.31	154.89 ± 1.53	154.11 ± 1.43	155.97 ± 1.00	155.35 ± 3.16
Absolute liver weight (g)	5.26 ± 0.12	5.45 ± 0.18	5.64 ± 0.11*	5.90 ± 0.10**	5.46 ± 0.16	5.56 ± 0.11
Relative liver weight[a]	3.35 ± 0.08	3.50 ± 0.12	3.64 ± 0.08*	3.88 ± 0.07*	3.50 ± 0.08	3.58 ± 0.08

Note: Values are means ± SEM.

[a] Relative liver weight = (absolute liver weight/terminal body weight) × 100.
Standard error of the mean (SEM) = standard deviation (S.D.)/√total subject.

* Significant difference at $P < 0.05$ level, when compared with the control group.

** Significant difference at $P < 0.01$ level, when compared with the control group.

Table 10 – Histopathological findings in male and female rats treated with prulifloxacin for treatment (28 days; $n = 12$ in each group) and recovery (14 days; $n = 6$ in each group) at the doses (mg/kg/day).

	After treatment period								After recovery period			
	Male				Female				Male		Female	
	0	100	200	400	0	100	200	400	0	400	0	400
Heart												
Cardiomyopathy	0	2	1	3	0	0	2	2	0	1	0	1
Cytoplasmic vacuolation	1	0	1	2	0	0	2	1	0	0	0	1
Liver												
Microgranuloma	0	1	4	8	1	0	3	4	0	1	0	1
Focal inflammation	0	0	2	2	0	0	3	2	0	1	0	0
Effacement of liver architecture	0	1	2	4	0	0	2	3	0	1	0	1
Derangement of hepatocyte	0	0	3	4	0	3	2	5	0	1	1	1
Vascular prominences	0	1	1	7	0	1	1	6	0	0	0	1
Lung												
Focal hemorrhage with alveolus	0	1	2	2	1	0	3	3	0	1	0	0
Kidney												
Hydronephrosis	0	1	1	4	0	1	1	3	0	0	0	1
Protein cast	1	1	2	3	0	1	2	2	1	2	0	1
Pyelonephritis	1	0	2	2	0	0	1	2	0	0	0	2
Stomach												
Focal ulceration in gastric mucosa	0	4	4	6	0	1	4	4	0	1	0	1
Nutrifilic infiltration in mucosa	0	0	4	2	0	1	1	2	1	0	1	1
Pancreas												
Degeneration of exocrine gland	0	0	2	1	1	1	2	2	0	1	0	1
Lymphoid cell infiltration	0	0	2	2	0	0	2	2	0	1	0	1
Achilles tendon												
Degenerated tendinocytes	1	0	0	0	0	0	1	1	0	1	0	0
Chrondrocyte-like tendinocytes	0	0	1	1	1	1	1	2	0	1	0	0
Rupture at insertion areas	0	1	1	2	1	0	1	1	0	1	0	0
Vascular proliferation	1	1	2	1	0	1	2	1	0	1	0	1
Talar joint												
Articular proteoglycan loss	0	1	4	3	1	4	4	6	0	1	0	1
Snovial infiltrate	0	2	1	4	0	1	2	6	0	1	0	1
Eroding cartilage	0	1	3	6	0	2	1	5	0	0	0	2
Salivary gland												
Atrophy of gland	0	1	2	2	0	0	0	1	0	0	0	0
Fibrosis	0	0	0	2	0	1	0	2	1	0	0	0
Testis												
Tubular atrophy	1	1	5	1					0	0		
Spermatide retention	1	0	2	6					0	2		
Prostate												
Atrophy	1	1	3	2					0	2		
Ovary												
Luteal cyst					0	1	2	3			0	0

treated with PRF after treatment and recovery period are illustrated in Table 9. After recovery period these differences in organ weights ware no longer seen.

3.9. *Histopathology*

Results of histopathological examination are illustrated in Table 10. Upon microscopic examination, mild to moderate lesions in treatment groups, erosion of the articular surface of talar joint, mild hydropic or fatty degeneration of the liver, vascular changes in pulmonary arteriole, intestinal inflammation and focal ulceration in gastric mucosa were detected sporadically in a dose-dependent manner at the end of treatment period.

Histopathology of talar joint showed that articular cartilage proteoglycan loss and synovial infiltrate adhering to and eroding cartilage in both sex at the end of treatment period (Fig. 4). Histopathological study of liver (nine male and five female rats of high dose group) shows severe hepatic injury, partial effacement of liver architecture, mild hydropic or fatty degeneration in the liver and inflammatory cell infiltration in the hepatic lobule of portal area (Fig. 5). Histopathological study of stomach (seven male and four female rats of high dose group) also shows focal ulceration in gastric mucosa

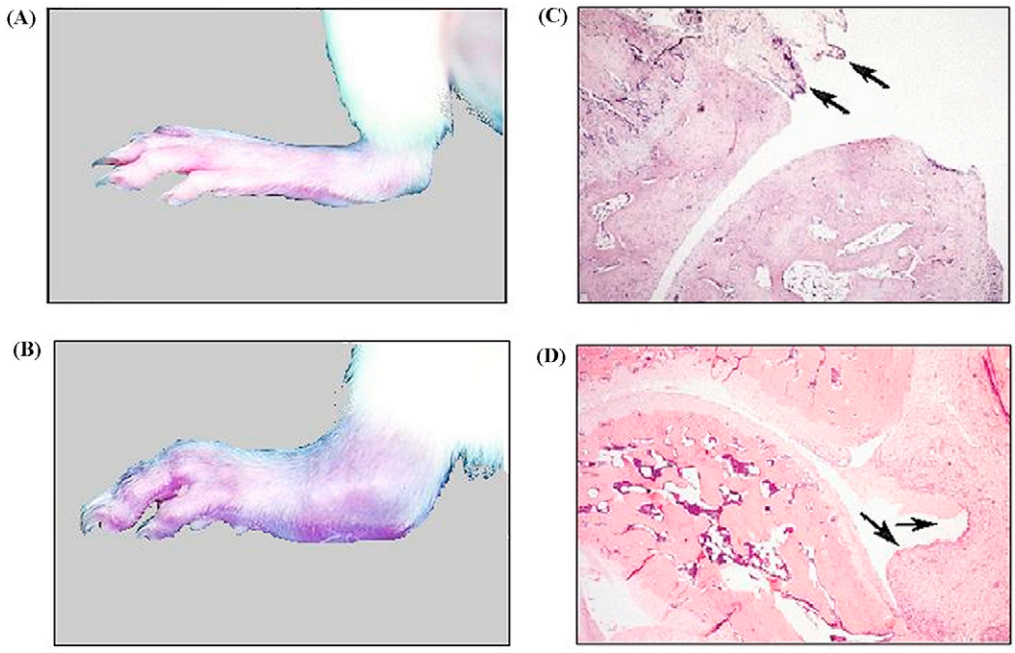

Fig. 4 – Photographic and histological features of the talar joint of hindlimb of female rat's (A, C) in vehicle control and (B, D) prulifloxacin treated group (400 mg/kg/day). (A) Photographic picture of normal hindlimb. (B) Photographic picture of hindlimb with swelling talar joint. (C) Histopathology of talar joint showing the normal non-arthritic joint with thin, paucicellular synovium (arrows) (HE 400×). (D) Histopathology of talar joint showing articular proteoglycan loss (arrow) and synovial infiltrate adhering to and eroding cartilage (arrow) (HE 400×).

and fair amount of neutrophil infiltration within mucosa and muscular wall (Fig. 6). Histopathology of lung showed (eight male and six female rats of high dose group) vascular changes in pulmonary arteriole, mild medial hypertrophy and marked intimal hyperplasia, leading to partial obstruction of the lumen (Fig. 7).

3.10. *Toxicokinetics and tissue distribution study*

Initially, validation was performed to evaluate the calibration, accuracy and precision of ULF in different tissues along with plasma. Calibration curve was constructed by plotting the peak area ratio (*f*) of ULF to IS *versus* the nominal concentration (*C*) of the ULF. The regression equation of the calibration curve was then used to calculate the concentration of ULF in different tissues and plasma. Back-calculated values of the concentrations were statistically evaluated. Back-calculated concentrations (mean ± S.D.) from the representative calibration standards by LC–MS/MS determination for ULF and corresponding regression equations are given in Table 11. This method also showed good linear responses over the selected concentration range in tested matrixes of rat. Additional evaluation of the lower limit of quantitation (LLOQ) confirmed that those concentrations fitted with the proposed criteria.

Typical MRM chromatograms of blank rat plasma (free of analyte and IS), rat plasma spiked with analyte (ULF) at LLOQ and rat plasma spiked with IS (25 mL of 1 mg/mL equivalent to 0.025 mg) are presented in Fig. 8. Representative chromatograms of blank rat liver (free of analyte and IS), rat liver spiked with analyte (ULF) at LLOQ and rat liver spiked with IS (25 mL of 1 mg/mL equivalent to 0.025 mg) are presented in Fig. 9. It can be shows from these figures, no significant interfering peaks were observed at retention times of analyte and/or IS. There were no significant differences in pharmacokinetic parameters of ULF between males and females during 28-days oral administration for three different doses. Therefore, we combined the pharmacokinetic parameters from male and female rats together. Values of the kinetic variables that described absorption and disposition kinetics of ULF in rats were determined (Table 12). The time course changes of the concentration of oral PRF administration in Wistar albino rats are shown in Fig. 10. Systemic exposure to PRF was indicated by the plasma C_{max} and AUC values over 24 h dosing at different time points.

Mean plasma concentration–time profiles showed that exposure to PRF increased as the target dose level increased from 100 to 400 mg/kg/day in day-1 and -28, respectively. An increase of exposure with time in terms of C_{max} and AUC values at the middle and high dose levels. At 400 mg/kg/day dose group, mean $AUC_{0-24\,h}$ value increased from 26.10 μg h/mL at day 1 to 31.24 μg h/mL at day 28, and at 200 mg/kg/day dose group, mean AUC value increased from 17.98 μg h/mL at day 1 to 23.75 μg h/mL at day-28. Dose normalize plots are shown in Fig. 11. Tissue distribution of ULF after 28 days oral dosing in Wistar albino rats is shown in Fig. 12. Tissue accumulation after recovery period (42nd day) in Wistar albino rats is shown in Fig. 13.

Table 11 – Liner regression data for the determination of ulifloxacin added to rat tissue homogenates and plasma[a] ($n = 6$).

Matrixes		Spiked concentration (μg/mL)										Equation	r^2
		0.01	0.025	0.05	0.1	0.25	0.5	1.0	1.5	2.0	2.5		
Plasma	Mc	0.008 ± 0.001	0.028 ± 0.007	0.041 ± 0.005	0.087 ± 0.009	0.268 ± 0.042	0.523 ± 0.095	0.983 ± 0.084	1.495 ± 0.140	2.001 ± 0.431	2.501 ± 0.589	$f = 2.6627C + 0.0506$	0.9998
Liver	Mc	0.009 ± 0.002	0.026 ± 0.005	0.036 ± 0.002	0.094 ± 0.007	0.244 ± 0.051	0.552 ± 0.004	1.072 ± 0.108	1.501 ± 0.233	1.952 ± 0.162	2.412 ± 0.601	$f = 1.2701C + 0.101$	0.9924
Lung	Mc	0.006 ± 0.004	0.031 ± 0.002	0.041 ± 0.003	0.091 ± 0.002	0.262 ± 0.044	0.507 ± 0.010	0.994 ± 0.051	1.796 ± 0.301	2.148 ± 0.120	2.433 ± 0.452	$f = 2.7598C + 0.076$	0.9889
Kidney	Mc	0.006 ± 0.001	0.029 ± 0.004	0.039 ± 0.002	0.086 ± 0.001	0.247 ± 0.036	0.475 ± 0.025	0.938 ± 0.049	1.694 ± 0.298	2.026 ± 0.320	2.393 ± 0.510	$f = 2.8238C + 0.050$	0.9928
Heart	Mc	0.009 ± 0.002	0.031 ± 0.001	0.043 ± 0.003	0.088 ± 0.004	0.243 ± 0.029	0.483 ± 0.051	0.939 ± 0.033	1.677 ± 0.150	2.008 ± 0.415	2.418 ± 0.298	$f = 2.8249C + 0.040$	0.9942
Stomach	Mc	0.015 ± 0.003	0.034 ± 0.002	0.054 ± 0.002	0.100 ± 0.002	0.261 ± 0.017	0.486 ± 0.022	0.924 ± 0.062	1.497 ± 0.177	2.112 ± 0.235	2.442 ± 0.435	$f = 2.891C + 0.0007$	0.9971
Intestine	Mc	0.008 ± 0.001	0.028 ± 0.004	0.052 ± 0.001	0.098 ± 0.001	0.244 ± 0.009	0.473 ± 0.062	0.898 ± 0.010	1.521 ± 0.104	2.106 ± 0.412	2.417 ± 0.401	$f = 2.8375C - 0.011$	0.9960
Testes	Mc	0.007 ± 0.004	0.018 ± 0.003	0.045 ± 0.003	0.088 ± 0.002	0.230 ± 0.011	0.453 ± 0.084	0.869 ± 0.071	1.481 ± 0.207	2.037 ± 0.189	2.468 ± 0.662	$f = 2.883C - 0.0331$	0.9978
Ovaries	Mc	0.012 ± 0.002	0.024 ± 0.001	0.051 ± 0.002	0.109 ± 0.004	0.248 ± 0.008	0.502 ± 0.022	0.871 ± 0.038	1.467 ± 0.075	2.170 ± 0.245	2.429 ± 0.424	$f = 2.959C - 0.0366$	0.9929
Spleen	Mc	0.009 ± 0.003	0.028 ± 0.003	0.054 ± 0.005	0.117 ± 0.006	0.256 ± 0.024	0.485 ± 0.038	0.820 ± 0.025	1.467 ± 0.075	2.092 ± 0.328	2.581 ± 0.333	$f = 3.264C - 0.1372$	0.9904
Brain	Mc	0.010 ± 0.001	0.023 ± 0.002	0.048 ± 0.001	0.078 ± 0.002	0.212 ± 0.030	0.442 ± 0.011	0.775 ± 0.020	1.318 ± 0.042	1.749 ± 0.184	2.241 ± 0.521	$f = 2.448C - 0.0133$	0.9927
ADP	Mc	0.008 ± 0.002	0.027 ± 0.003	0.051 ± 0.002	0.010 ± 0.004	0.252 ± 0.011	0.532 ± 0.028	0.848 ± 0.101	1.417 ± 0.029	1.947 ± 0.222	2.634 ± 0.382	$f = 3.099C - 0.0866$	0.9924

S.D., standard deviation; f, mean peak area ratio of ulifloxacin to IS; r^2 (concentration coefficient), linearity of the calibration curve; Mc, measured concentration (ng/mL); ADP, adipose tissue.
[a] Measured concentration (Mc) presented by mean ± S.D.

Fig. 5 – Representative histopathological picture of male rat's liver (A) in vehicle control, (B) prulifloxacin treated group (400 mg/kg/day). (A) Histopathology of liver showing the normal architecture and cells with granulated cytoplasm and small uniform nuclei (HE 200×). (B) Histopathology of liver showing the severe hepatic injury, partial effacement of liver architecture, mild hydropic or fatty degeneration in the liver and inflammatory cell infiltration in the hepatic lobule of portal area (HE 400×).

4. Discussion

Present study was conducted to determine the potential repeated dose toxicity, tissue distribution and toxicokinetics of ULF during 28-days oral administration of PRF. Reversibility of its toxic effects, tissue accumulation of ULF after recovery period, and thus to investigate the correlation between oral dose toxicity, toxicokinetics and tissue distribution of ULF in Wistar albino rats in same experiment. Here we have compared the observed histology and findings with previously reported literature for PRF and other fluoroquinolones. Results showed that exposure of Wistar albino rats to repeat dose of PRF produced a number of treatment-related effects, particularly in the high dose group.

Food consumption temporarily was suppressed in the 400 mg/kg/day dose group of both sex but returned toward

Table 12 – Toxicokinetic parameters (mean ± SEM) of ulifloxacin in rat plasma following repeated oral dosing of (100, 200 and 400 mg/kg/day) prulifloxacin ($n = 24$).

Toxicocokinetic parameters	Rat plasma					
	1st day			28th day		
	100	200	400	100	200	400
C_{max} (μg/mL)	1.099 ± 0.027	1.463 ± 0.021	1.992 ± 0.110	1.354 ± 0.022	1.881 ± 0.017	2.589 ± 0.016
T_{max} (h)	6.333 ± 0.225	6.000 ± 0.000	6.000 ± 0.000	5.833 ± 0.297	6.000 ± 0.246	5.833 ± 0.167
$t_{1/2}$ (h)	14.527 ± 1.605	13.681 ± 0.938	16.712 ± 0.643	14.566 ± 1.066	13.153 ± 0.655	15.370 ± 0.359
$AUC_{0-24\,h}$ (h μg/mL)	15.411 ± 0.755	17.986 ± 0.534	26.101 ± 1.575	19.067 ± 0.278	23.758 ± .264	31.245 ± 1.020
$AUC_{0-\infty}$ (h μg/mL)	24.083 ± 2.333	29.076 ± 3.132	34.089 ± 2.386	30.452 ± 1.695	35.977 ± 1.356	43.843 ± 1.917
K_{el}	0.064 ± 0.010	0.059 ± .007	0.052 ± 0.001	0.050 ± 0.003	0.054 ± 0.002	0.061 ± 0.002

C_{max}, maximum plasma concentration; t_{max}, time require to achieve maximum concentration; AUC_{0-24}, area under the plasma concentration time curve from time zero to 24 h; $AUC_{0-\infty}$, plasma concentration–time curve from time zero to infinity; $t_{1/2}$ (h), elimination half life; K_{el}, elimination rate constant.

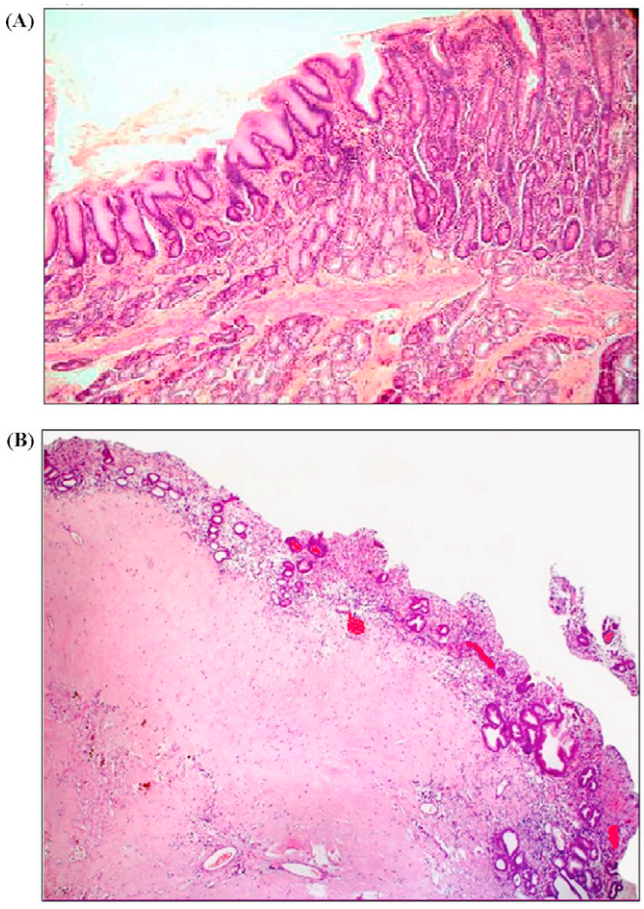

Fig. 6 – Representative histopathological picture of male rat's stomach (A) in vehicle control and (B) prulifloxacin treated group (400 mg/kg/day). (A) Histopathology of stomach showing the normal architecture and the mucosa are lined by simple columnar epithelium cells with granulated cytoplasm (HE 200×). (B) Histopathology of stomach showing the focal ulceration in gastric mucosa and muscular wall (HE 200×).

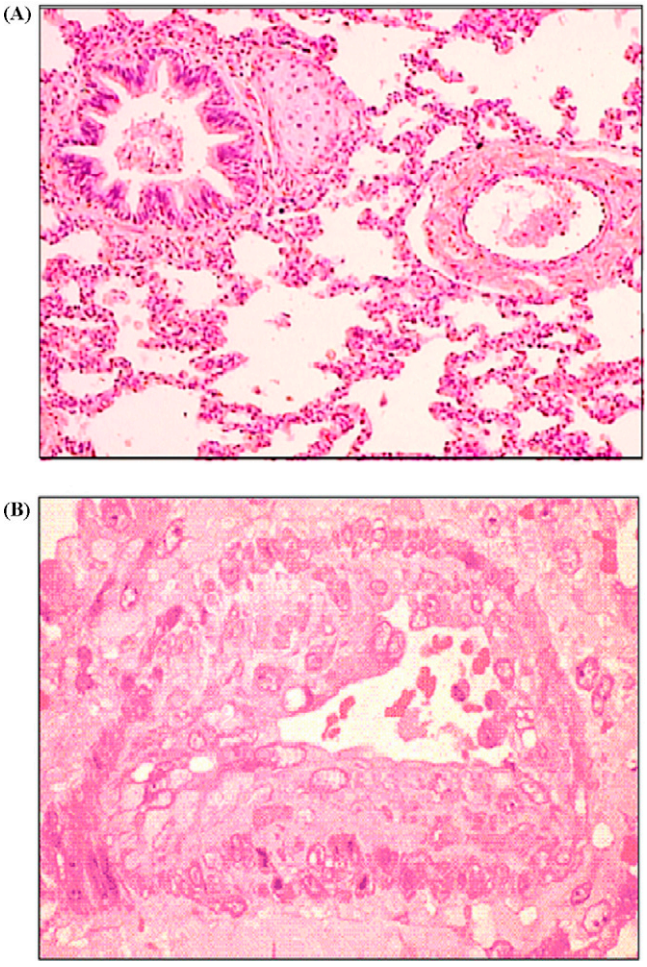

Fig. 7 – Representative histopathological picture of male rat's lung (A) in vehicle control and (B) prulifloxacin treated group (400 mg/kg/day). (A) Histopathology of lung showing the normal architecture and the alveoli are composed of single layer of squamous epithelium e (HE 400×). (B) Histopathology of lung showing the vascular changes in pulmonary arteriole, mild medial hypertrophy and marked intimal hyperplasia, leading to partial obstruction of the lumen (400 mg/kg/day).

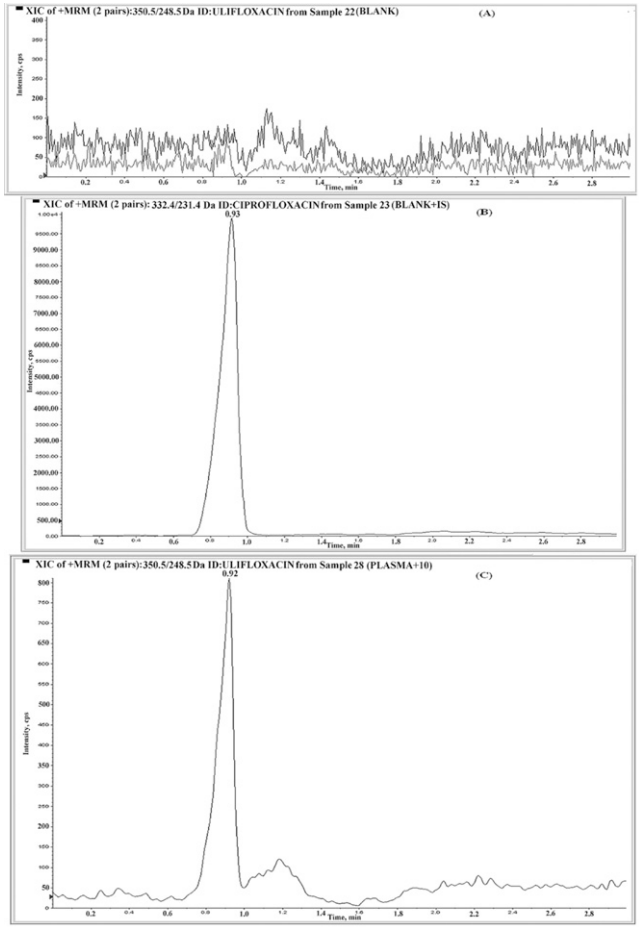

Fig. 8 – Typical MRM chromatograms of (A) blank rat plasma (free of analyte and IS), (B) rat plasma spiked with IS (25 μL of 1 μg/mL equivalent to 0.025 μg) and (C) rat plasma spiked with analyte (ULF) at LLOQ.

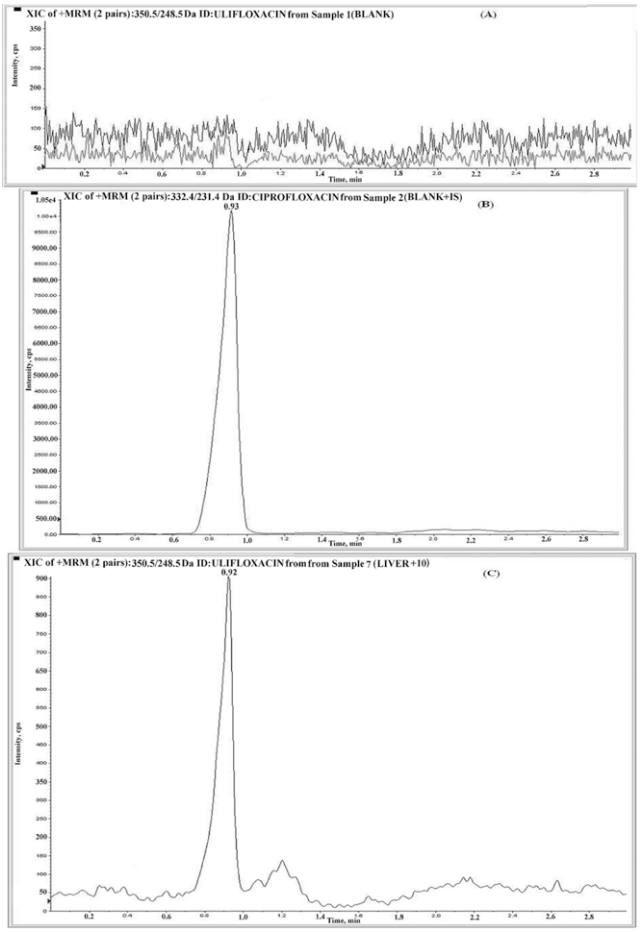

Fig. 9 – Typical MRM chromatograms of (A) blank rat liver (free of analyte and IS), (B) rat liver spiked with IS (25 μL of 1 μg/mL equivalent to 0.025 μg) and (C) rat liver spiked with analyte (ULF) at LLOQ.

normal levels during recovery period. This appetite suppression was probably due to direct irritation of the gastrointestinal lining. Mean body weight of animals in the high dose group was significantly lower than the weight of other groups throughout the treatment period of repeated oral dose toxicity study. Effect on body weight was not extreme, but suggests that 400 mg/kg/day PRF treatment had a sustained, suppressive influence on growth. Repeated oral dosing produced a marked increase in relative liver weight (male, 16.56%; female, 15.82%), which was higher than control value for the high dose group. A possible explanation for the increased liver weights of high dose group of rats could be the induction of hepatic detoxification enzymes such as cytochrome P450 (Woodman, 1996), a mechanism currently being investigated. Serum hematological analysis at the end of treatment period (after 28-days) of repeated oral dose toxicity study also indicate that significant increase of serum total WBC count (male, 50.29%; female, 58.55%) in high dose group of both sex is closely related to the administration of PRF, and showed a dose–response relationship. Increase of serum total WBC counts resulted also from the elevation of lymphocytes. These findings similar to the previous studies of PRF and other fluoroquinolones in rats (Yoshida et al., 1996; Nishimura et al., 1996; Oda et al., 1996; Roy et al., 2010a,b).

In repeated oral dose toxicity study, dose-dependence on significant increase in serum T-BIL, glucose, serum ALT and significant decrease of serum TP in the 400 mg/kg/day dose group (in both sex) suggest that these findings were related to the administration of PRF and were linked with increase of liver weight with intestinal inflammation. This finding is consistent with the results of previous reports showing that a number of fluoroquinolones induced digestive system damage (Ball, 2003; Von Keutz and Schluter, 1999) and liver damage (Shimada and Hori, 1992; Von Keutz and Schluter, 1999; Guzman et al., 2000) in both humans and experimental animals. However, all values returned toward normal levels following the recovery period of 14-days, indicating that the toxic effects are transient and reversible.

In this study, synovial fluid of talar joint (right hindlimb) showed dose-dependence on significant increase of TNCC and neutrophils, LDH, UA and TP and the significant decrease of glucose concentration in 400 mg/kg/day dose group (in both sex), which suggest that findings were related to the

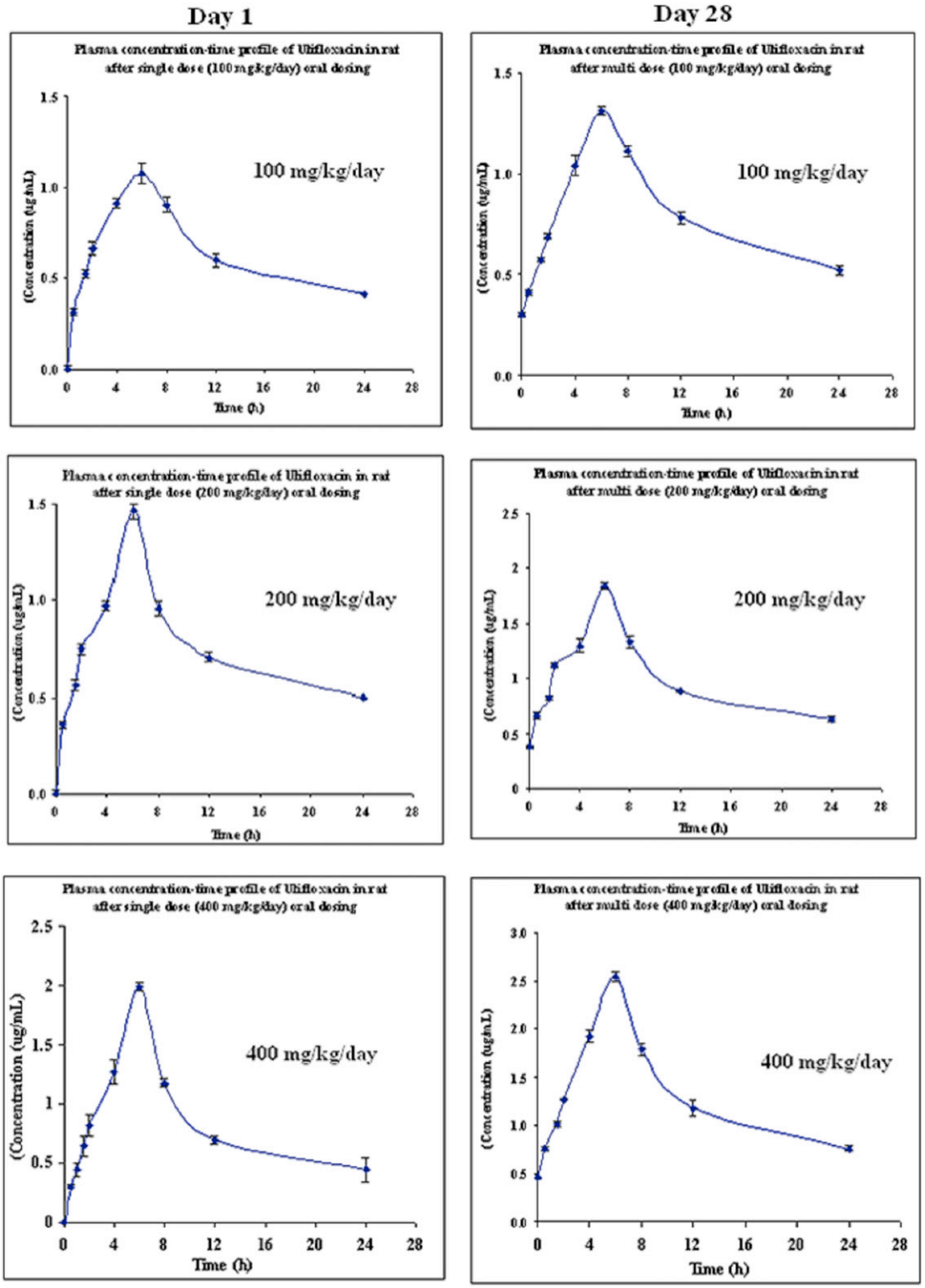

Fig. 10 – Mean plasma concentration–time profiles of prulifloxacin during a repeated-dose toxicity study in Wistar albino rats. Serial blood samples (0.5, 1.5, 2, 4, 6, 8, 12 and 24 h) were collected from the retro orbital sinus of animals receiving oral doses of 100, 200 or 400 mg/kg/day of prulifloxacin, on days 1 and 28 during the course of the repeated-dose toxicity study.

administration of PRF and were linked with the joint inflammation, infection, joint damage and arthritis or gout. Presence of MSU and CPPD crystal in synovial fluid in high dose group (400 mg/kg/day) in both sex suggest that findings were related to the administration of PRF and were linked with joint damage and gout or pseudo-gout.

Some abnormal histopathological changes in liver, stomach and lung architecture were also observed in some rats

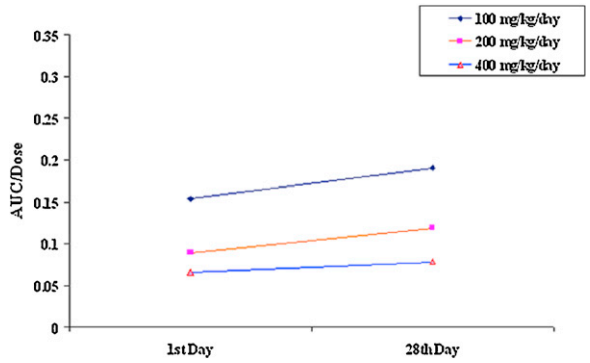

Fig. 11 – Dose normalized plots for AUC.

of high dose group (in both sex). Adverse effects on liver including elevated transaminases, alkaline phosphatase and bilirubin concentrations and histological changes due to fluoroquinolones exposure have already been reported in human and experimental animals (Shimada and Hori, 1992; Von Keutz and Schluter, 1999; Guzman et al., 2000; Roy et al., 2010a,b). In this study, phototoxic potentials of PRF were determined systemically. At 400 mg/kg/day oral dose of PRF produced severe ear erythema and redness of the ears even after 7 days of treatment in both sex. Actual reason is unknown at this time, but result of this study clearly express that PRF may have some phototoxic effect on rats as like other fluoroquinolones (Prats et al., 2006).

Our study on the toxicokinetics of PRF in rats provides an insight into the time course of concentration in different biological samples. Appearance of ULF in plasma and tissues of rat was characterized by a slow but well distribution phase, followed by a slower elimination phase. The $t_{1/2}$ of ULF on day-1 in 400 mg/kg/day dose group (16.71 ± 0.643 h) was similar to that observed for ULF in previous study (16.712 ± 2.22 h) (Roy, 2010), but longer to that of day-28 (15.37 ± 0.35 h). Increase in C_{max} and $AUC_{0-24\,h}$ values were approximately proportional to the increase in the dose level during both collection intervals and no saturation was observed. At the 200 and 400 mg/kg/day dose level, higher exposures were noted on day-28 as compared to day-1, which suggests that ULF is highly accumulated. Fig. 11 shows slightly higher $AUC_{0-24\,h}$ on day-28 at the 200 and 400 mg/kg/day dose level, which means higher exposures on day-28 as compared to day-1 and suggests the presence of little induction occurring during absorption of high dose. Since there is not much known about the transport process in absorption of PRF, this remains speculative at this point. Some non-linear kinetics of ULF was observed. It was shown that ULF was distributed to the major organs after oral administration and concentration in lung nearly equivalent to the plasma concentration. ULF concentrations were high in the lung, liver, heart, kidney, testes, and moderate in the stomach, spleen, intestine, ovaries and low in the adipose tissue and brain. This study serves to better understand the rodent toxicity of PRF and to design further studies to characterize toxicity of PRF in higher mammalian and human. Apart from lung, liver, kidney and testes no significant accumulation of ULF in organs and tissues were observed after recovery period (42nd day).

Based on these results, it was concluded that long-term repeated dose of PRF may produce different blood parameters abnormality, liver damage, stomach ulcer, joint damage and dysfunction of lung in Wistar albino rats. One of the most common reasons behind of these types of effect is high tissue distribution & accumulation of ULF in these tissues. After oral administration, PRF is absorbed in the upper small intestine and then metabolized to ULF, mainly by paraoxonase enzyme

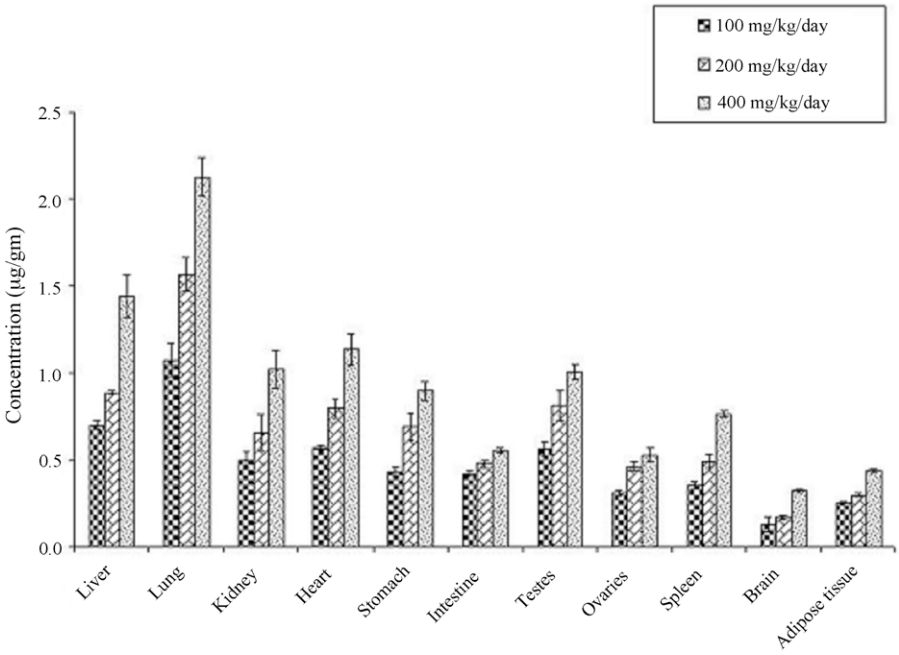

Fig. 12 – Tissue distribution of ulifloxacin in Wistar albino rats at 24 h (28th day) after a repeated-dose of 100, 200 or 400 mg/kg/day of prulifloxacin.

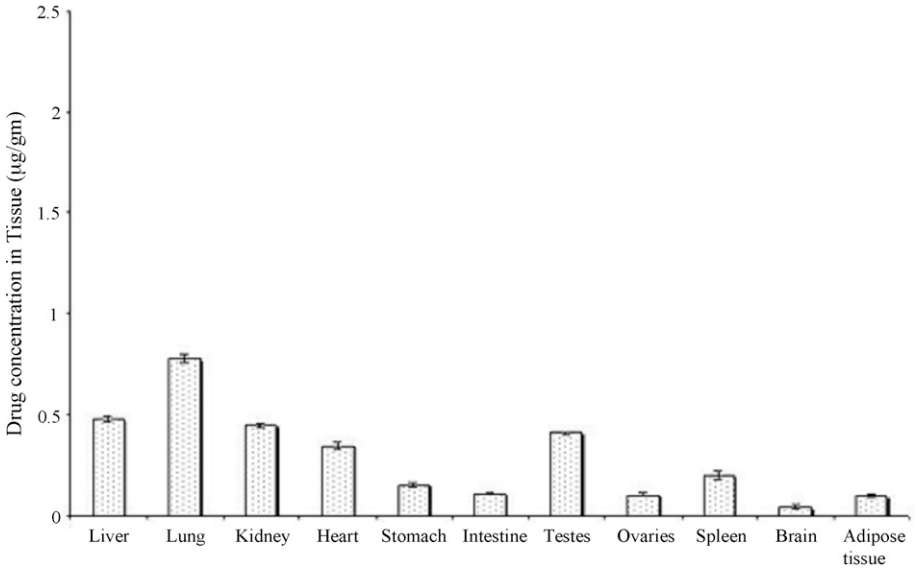

Fig. 13 – Tissue accumulation of ulifloxacin in Wistar albino rats after 14 days recovery period (42nd day) at 400 mg/kg/day group.

and distributed to the many tissues along with blood plasma by following some non-linear kinetics. Lung tissue specificity of ULF is very high and in lung tissue concentration of ULF is nearly equivalent to the plasma concentration. Along with lung, ULF was also well distributed in liver, heart, kidney and other organs. For this reason PRF may give prolong action after oral administration and produce some adverse effect in different organs. Therefore, clinical efficacy of PRF cannot just be predicted relating to the serum concentration to minimum inhibitory concentration (MIC) and attention should be paid to tissue pharmacokinetic concept. Clinical effects can still observe due to the penetration of PRF into target tissues, even plasma concentration fallen below MIC. These findings may have an impact on the management of PRF induced adverse effects to serve as a pre-clinical database for appropriate dose selection in clinical practice.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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