

Determination of renal handling of marbofloxacin in Lohi sheep (*Ovis aries*) following a single intravenous administration

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Summary

The objective of present study was to investigate renal clearance, urinary excretion and underlying excretory mechanism of marbofloxacin in Lohi sheep. For this purpose, marbofloxacin was administered intravenously (IV) as single bolus dose (2.5 mg/kg body weight) to eight healthy sheep of Lohi breed. After start of experiment, blood and urine samples were drawn at predetermined time intervals and marbofloxacin concentrations in the samples were measured by reverse phase high performance liquid chromatography (RP-HPLC) using UV/Vis detector. The mean \pm SD values of creatinine in plasma and urine were 15.37 ± 0.65 $\mu\text{g/ml}$ and 246.7 ± 48.05 $\mu\text{g/ml}$, respectively. Glomerular filtration rate was 1.29 ± 0.22 ml/min/kg whereas urinary flow rate was observed to be 0.084 ± 0.016 ml/min/kg. The renal clearance of marbofloxacin in Lohi sheep was 9.45 ± 2.12 ml/min/kg. Cumulative percentage dose excreted was seen to be maximum at 24 h post drug administration. It was concluded that renal handling of marbofloxacin in Lohi sheep involved both glomerular filtration and active tubular secretion.

Key words: HPLC, Lohi sheep, Marbofloxacin, Renal clearance, Urinary excretion

Introduction

Like many other developing countries, Pakistan also imports pharmaceutical raw material or finished products, especially the antimicrobials, for its health programs both in humans and veterinary medicine. Environmental conditions, being different in Asian and European countries, have an impact on the genetic makeup of human and livestock population, hence a change in the genetic makeup is observed among the population of different countries (Iqbal *et al.*, 2011, 2012). In addition, under local circumstances, the pharmacokinetic data of investigated drugs were dissimilar to the values provided by the manufacturing companies (Javed *et al.*, 2003; Muhammad *et al.*, 2003; Iqbal *et al.*, 2015; Manzoor and Iqbal, 2016) on the basis of which it is hypothesized that renal handling of marbofloxacin in sheep of Lohi breed is different from those of its foreign counterparts and other animal species.

Marbofloxacin is a broad spectrum antibacterial agent which is extensively used against different microbial infections in the food producing animals. Different analytical methods like spectrophotometer, microbiological assay and chromatographic assay are commonly employed to determine the concentration of a drug in plasma and urine. However, some discrepancies have been observed between the obtained results of these techniques due to the presence of active metabolites of parent drug (Garcia *et al.*, 1999). HPLC is the most

appropriate technique to quantify the drug concentration in biological fluids. Although marbofloxacin is very an important antimicrobial agent for veterinary use, very limited data are available regarding its quantification and underlying renal handling mechanisms in urine of sheep and especially Lohi breed stressing on the need to develop a specific, cost effective and rapid method for marbofloxacin determination in urine of sheep. Hence, the present study was conducted to investigate renal clearance, urinary excretion and underlying excretory mechanism of marbofloxacin in Lohi sheep using RP-HPLC method.

Materials and Methods

Animals

Eight healthy adult female sheep of Lohi breed (native to Pakistan) were selected for this study. The weight of sheep ranged between 37-48 kg. Animals were housed at the Livestock Experimental Farm, Institute of Nutrition & Feed Technology, University of Agriculture, Faisalabad and care was taken according to the national animal welfare guidelines. Prior to the study, each animal was critically examined for any sign of disease. Each animal was kept in a separate pen to avoid any physical contact with other animals. Seasonal green fodder was provided to all the animals *ad libitum* and they had free access to water all the time. The study protocol was critically reviewed and approved by the

Institutional Review Board and Ethics Committee of Isra University, Islamabad vide notification No. 3-2/IUIC/ANMC/CE-18/2012 dated 31st May, 2012.

Renal clearance

A plastic cannula No. 90 (Protex Ltd., England) was used to cannulate left jugular vein of each animal aseptically. Each animal was catheterized with sterilized disposable Foley's balloon catheter (Rush No. 14, 30 ml, Henso, China). For the collection and measurement of urine, a calibrated reservoir was applied to the external opening of each catheter.

Administration of drug and collection of blood samples

Marbofloxacin (CAS, 115550-35-1) was procured from Hanqzou Thick Chemical Co. Ltd., China and injected as a single intravenous dose of 2.5 mg/kg body weight in the right jugular vein of each animal (Sidhu *et al.*, 2010; Karademir *et al.*, 2015). Three ml of blood was collected at 0.5, 1, 1.5, and 2.0 h in heparinized plastic centrifuge tubes for the calculation of renal clearance. The pH of each blood sample was measured by an electronic pH meter (Beckman HS, Germany). Blood samples were centrifuged at 4000 rpm for 15 min to separate plasma which was then stored at -4°C till analysis.

Collection of urine samples

For collecting urine samples, urinary bladder of each animal was completely emptied and washed with distilled water at 45 min post drug administration. Urine samples were collected at 75, 105, 135, and 165 min post drug administration for the determination of renal clearance of marbofloxacin. Further urine samples were collected at 4, 8, 12, and 24 h to calculate the cumulative percentage dose excreted in urine. Marbofloxacin concentration in plasma and urine was quantified by RP-HPLC method according to the technique described by Garcia *et al.* (1999) with few modifications. These modifications in the HPLC system were in detector being different in type and in column being different in dimensions.

Chemicals

Marbofloxacin mesylate (Hanqzou Thick Chemical Co. Ltd., China) and sarafloxacin (Ferozsans Pvt. Ltd., Pakistan) served as external and internal standards (IS), respectively. Orthophosphoric acid, acetonitrile, disodium hydrogenphosphate, potassium dihydrogen phosphate and trichloromethane were purchased from Scharlau (Barcelona, Spain). Tetraethylammonium bromide was obtained from Sigma Chemical Co. (St-Louis, USA). All the chemicals were of analytical grade.

Standard solutions

Each day stock solutions of marbofloxacin and sarafloxacin were prepared in water (0.1 mg/ml). These

solutions were spiked to drug free plasma of Lohi sheep in order to determine the recovery, precision, accuracy and detection limit. All standards were wrapped with aluminium foil in order to protect them from light and kept at 4°C until use.

Extraction procedure

Aliquots (200 ml) of plasma samples were diluted with 800 ml of 0.1 M phosphate buffer (pH = 7.4) containing 1500 ng/ml of sarafloxacin as the internal standard. 6 ml of trichloromethane was added to these samples and shaken at 200 oscillations/min for 30 min. The samples were centrifuged at 13000 rpm for 6 min. The aqueous layer of the solution was removed and organic layer was transferred into a fresh tube and dried under a stream of nitrogen at 40°C. The residue was dissolved in 200 ml of phosphate buffered saline (PBS) and an aliquot (10-80 ml) was injected into the chromatographic system.

Instrumentation and chromatographic conditions

A High Performance Liquid Chromatograph (Model: Sykam, S-2210), which consisted of a stainless steel C₁₈ column (BDS, Thermo Hypersil, England) with specifications 250 × 4.6 mm and 5 µm particle size was applied to the system. This column was protected with a guard column (25 × 2.3 mm, Guard-PakTM, Waters, UK). The analytes were detected by a UV/Visible detector (Model: Sykam, S-5510). Computer software (Peak Simple Chromatography Data System, Buck Scientific Inc., East Norwalk) was used for the detection of output of the system.

Tetraethylammonium bromide (0.012 M), phosphoric acid (0.006 M) and potassium dihydrogenphosphate (0.020 M) were dissolved in distilled water to prepare aqueous solution. Acetonitrile was mixed with this aqueous solution (20:80, V/V) to prepare mobile phase. To remove any contamination, mobile phase was filtered through a 0.45 µm Lida filter before use. The UV detector was set at 275 nm. Analytes were allowed to flow at a rate of 1 ml/min into the system at 37°C, using an isocratic mode. The limit of quantitation (LOQ) was 0.25 µg/ml.

Calibration procedure

Appropriate volumes of stock solutions of marbofloxacin were spiked to sufficient amount of plasma in order to construct calibration curve. The final concentrations were in the range of 1-32 µg/ml. These calibration samples were then taken through the sample preparation procedure described above. The calibration curve was characterized by its regression coefficient, slope, and intercept, and it was used to determine the analyte concentrations in the samples and the detection limits. Finally, the sample concentrations were calculated by determining the peak height ratio of marbofloxacin to the internal standard, with these ratios being interpolated in the standard curves obtained for the calibration

samples.

Method validation

Linearity

Linearity of the method was assessed by the correlation coefficient of the calibration curves that were constructed from mean peak area of marbofloxacin at different concentration levels (80, 90, 100, 110, and 120% W/V solution of marbofloxacin in plasma blank).

Specificity

The standard solution of marbofloxacin was prepared and injected to the column. The retention time was observed. There were no interferences found.

Precision

The assay precision (relative standard deviation, RSD) was assessed by expressing the standard deviation of repeated measurements as a percentage of the mean value. Intra-day precision was estimated from 6 replicates of 3 standard samples used for calibration curves. Interday precision was estimated from the analysis of standard samples on 3 separate days.

Accuracy

The accuracy of the method was determined by comparing the measured concentration to its true value.

Recovery

The percentage recovery was estimated by successive analysis (n=3) for three different concentrations of standard solution. The data of the experiment were statistically analyzed using the formula [% Recovery = (Recovered conc./injected conc.) × 100] to study the recovery.

Creatinine determination in plasma and urine

The creatinine concentration was estimated by Jaffe reaction method in plasma and urine (Swenson, 1985). Creatinine clearance was considered as marker of glomerular filtration rate (GFR) in the current study.

Urinary excretion

Marbofloxacin was quantified as mentioned earlier. The pH of fresh urine samples was noted at the time of collection. At the specific time intervals (4, 8, 12, and 24 h post drug administration), the mean ± SD values for marbofloxacin were determined in the urine. Cumulative percentage of the dose excreted in the urine until 24 h post administration was determined by the following equations.

$$\text{Amount of drug excreted} = U_c \times U_v$$

where,

U_c: Concentration of drug in urine (mg)

U_v: Total volume of urine voided (ml)

$$\text{Percentage of dose excreted} = \frac{\text{Amount of drug excreted (mg)}}{\text{Total dose in (mg) given}} \times 100$$

$$\text{Percentage of cumulative dose excreted} = \frac{\text{Cumulative amount excreted (mg)}}{\text{Total dose of drug given}} \times 100$$

The renal clearance of creatinine and marbofloxacin was determined by the following equation (Swenson, 1985).

$$Cl_{\text{ren}} = \text{Concentration in urine} \times \text{urinary rate flow} / \text{plasma concentration}$$

In vitro plasma protein binding

In vitro binding of marbofloxacin to plasma proteins was estimated by equilibrium dialysis technique according to the method described by Kunin *et al.* (1959). Different concentrations of marbofloxacin (0.1, 0.5, 1, 5, and 10 µg/ml) were prepared in pooled plasma taken from untreated animals. Each dialyzing bag with a pore size of 4 Å was filled with 5 ml of plasma containing known amount of marbofloxacin. Each bag was then immersed in a separate tube containing 5 ml of phosphate buffer (0.2 M; pH = 7.4). These tubes were then incubated at 37°C for 24 h with occasional shaking. The concentration of marbofloxacin was separately analyzed in the buffer as well as in the contents of the dialyzing bags at the end of the incubation period. For each concentration three separate sets of experiments were conducted. The extent of *in vitro* plasma protein binding of marbofloxacin was calculated by the following equation (Ram *et al.*, 2008):

$$\text{Percent of levofloxacin bound to plasma protein} = \frac{CP' - CB}{CP} \times 100$$

where,

CP': The marbofloxacin concentration in plasma after incubation

CB: The marbofloxacin concentration in phosphate buffer after incubation

CP: The marbofloxacin concentration in plasma before incubation

The free drug concentration of marbofloxacin was plotted against the constant I_i which was obtained by the following equation:

$$I_i = P/T/L - W$$

where,

P: Protein content of plasma

T: Total concentration of drug

L: Free concentration of drug

W: Water content of plasma

β_i was calculated by least squares regression technique and its negative intercept with the ordinate was equal to K_β.

Statistical analysis

The mean ± SD values for plasma concentration at different time intervals were determined. The relationship between the renal clearance of marbofloxacin and plasma drug concentration, urinary flow rate and urinary pH was determined by regression correlation analysis using statistical software NCSS[®] version 9.

Results

A representative chromatogram of marbofloxacin standard in the urine of Lohi sheep is presented in Figs. 1a-b.

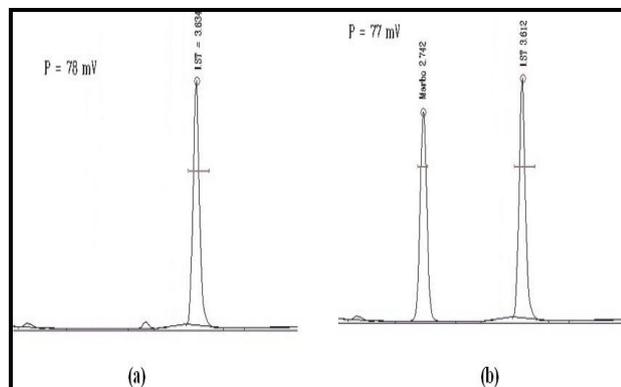


Fig. 1: Representative chromatograms of marbofloxacin and sarafloxacin. (a) Blank plasma spiked with 16 µg/ml of sarafloxacin, and (b) blank plasma spiked with 16 µg/ml of sarafloxacin and 16 µg/ml of marbofloxacin

For plasma, the method was linear with correlation coefficients (r) $>0.98\%$ for calibration curves and precise with RSD $<5.2\%$. Inter-day precision in terms of RSD $<6.3\%$. The intra-assay and interassay accuracies were $>96\%$ and $>97\%$, respectively. The mean percentage recovery of marbofloxacin from plasma was $95.14 \pm 1.48\%$. The limit of quantification (LOQ) was determined to be $0.25 \mu\text{g/ml}$.

For urine, the precision of the method with respect to RSD was $<4.6\%$. Inter-day precision with RSD was $<5.1\%$. The intra-assay and interassay accuracies were $>97\%$ and $>98\%$, respectively. The mean percentage

recovery was $97.63 \pm 1.13\%$. The limit of quantification (LOQ) and limit of detection (LOD) were observed to be 0.05 and $0.01 \mu\text{g/ml}$, respectively.

The respective results of diuresis, pH of blood and urine, renal clearance of creatinine and marbofloxacin are presented in Table 1.

The value of urinary flow rate was observed to be $0.084 \pm 0.016 \text{ ml/min/kg}$. The pH of blood was recorded as 7.85 ± 0.02 and for urine it was 7.86 ± 0.02 . The mean \pm SD values of creatinine were noted to be $15.37 \pm 0.65 \mu\text{g/ml}$ in the plasma of Lohi sheep, whereas $246.7 \pm 48.05 \mu\text{g/ml}$ of creatinine was recorded in urine. GFR was $1.29 \pm 0.22 \text{ ml/min/kg}$. The marbofloxacin plasma concentration was $0.28 \pm 0.01 \mu\text{g/ml}$ while it was calculated to be $30.09 \pm 2.33 \mu\text{g/ml}$ in urine. The renal clearance of creatinine and marbofloxacin was $1.29 \pm 0.22 \text{ ml/min/kg}$ and $9.45 \pm 2.12 \text{ ml/min/kg}$, respectively. The ratio of the marbofloxacin clearance to that of creatinine was estimated to be 7.58 ± 2.30 .

By regression correlation analysis, a significant ($P < 0.05$) negative correlation ($r = -0.48$) was observed between the plasma concentration of marbofloxacin and renal clearance ratio (Fig. 2), whereas urinary pH had very little influence ($r = 0.09$) on the clearance of drug (Fig. 3). Diuresis, on the other hand, showed very strong relation ($r = 0.47$) with the renal clearance of marbofloxacin in local sheep (Fig. 4).

The cumulative dose excreted at different time intervals is shown in Fig. 5. It is evident from Fig. 5 that the maximum dose was excreted at 4 h post drug administration.

At plasma concentrations of 0.1 - $10 \mu\text{g/ml}$, the extent of plasma protein binding of marbofloxacin ranged between 13.5% to 24.3% with an overall mean of $19.08 \pm 1.86\%$ (Table 2).

Table 1: The values for body weight, urinary flow rate, plasma concentration, urine concentration, renal clearance of creatinine (Cr), marbofloxacin (Marbo) and Marbo/Cr ratio in 8 sheep following intravenous administration of marbofloxacin at 2.5 mg/kg body weight

Animal #	Body weight (kg)	Urinary flow rate (ml/min/kg)	pH		Cr conc. ($\mu\text{g/ml}$)		Marbo conc. ($\mu\text{g/ml}$)		Renal clearance		
			Blood	Urine	Plasma	Urine	Plasma	Urine	Cr	Marbo	Marbo/Cr
1	39	0.098	7.84	7.82	15.39	264.1	0.31	29.83	1.63	9.56	5.71
2	41	0.115	7.86	7.88	16.29	171.7	0.26	29.95	1.14	13.96	12.24
3	40	0.079	7.82	7.89	15.63	284.7	0.29	31.8	1.45	9.73	6.71
4	38	0.081	7.85	7.85	14.84	231.7	0.29	30.28	1.23	9.12	7.85
5	44	0.065	7.87	7.88	14.16	275.5	0.27	25.03	1.25	6.33	4.97
6	37	0.088	7.86	7.85	15.26	229.4	0.28	29.6	1.29	9.3	7.73
7	42	0.072	7.87	7.87	15.92	198.1	0.31	32.83	0.88	8.32	9.21
8	48	0.07	7.83	7.87	15.5	318	0.27	31.43	1.42	9.32	6.29
Mean	41.1	0.084	7.85	7.86	15.37	246.7	0.285	30.09	1.29	9.455	7.589
SD	3.56	0.016	0.02	0.02	0.65	48.05	0.019	2.33	0.22	2.124	2.305

Table 2: Mean \pm SD *in vitro* marbofloxacin binding to plasma protein of Lohi sheep (each data point is average of 3 determinants)

Exp. No.	Extend of binding (%)					Association rate constant, β_1 (mol/g)	Dissociation rate constant, K_β (mol)
	Marbofloxacin concentration ($\mu\text{g/ml}$)						
	0.1	0.5	1	5	10		
1	13.4	16.8	18.6	21.2	22.7		
2	15.7	18.9	17.4	20.7	26.7		
3	11.6	14.7	20.8	23.4	23.7		
Mean \pm SE	13.56 \pm 1.18	16.8 \pm 1.21	18.93 \pm 0.99	21.76 \pm 0.82	24.36 \pm 1.20	2.1×10^{-8}	1.9×10^{-7}

Overall mean \pm SE of extent of binding (%) = 19.08 ± 1.86

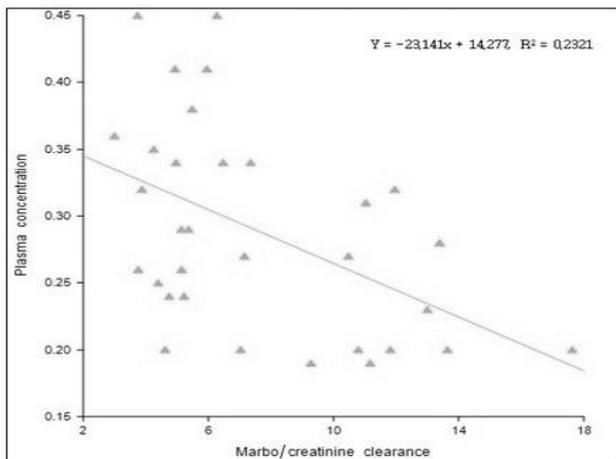


Fig. 2: Effect of plasma concentration of marbofloxacin on its renal clearance in Lohi sheep. Each point shows one of the 32 observations in 8 experiments, each comprised of 4 experimental periods

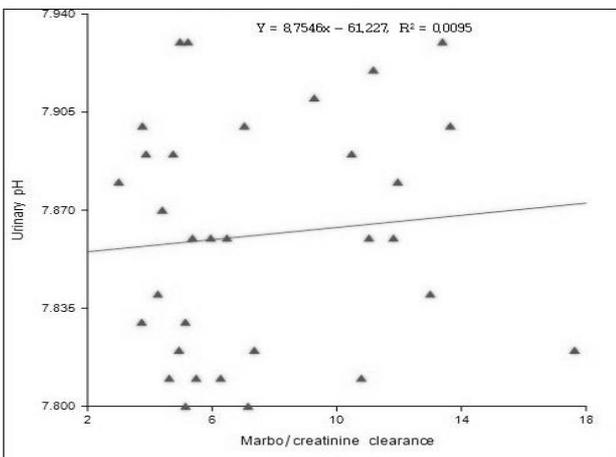


Fig. 3: Effect of urinary pH on renal clearance of marbofloxacin in Lohi sheep. Each point shows one of the 32 observations in 8 experiments, each comprised of 4 experimental periods

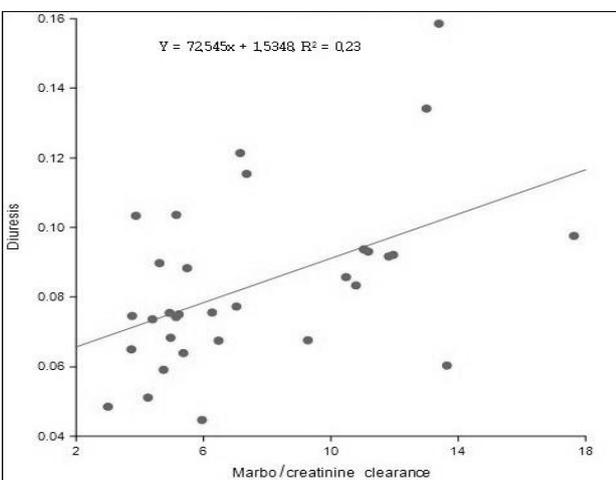


Fig. 4: Effect of urinary flow rate of marbofloxacin on its renal clearance in Lohi sheep. Each point shows one of the 32 observations in 8 experiments, each comprised of 4 experimental periods

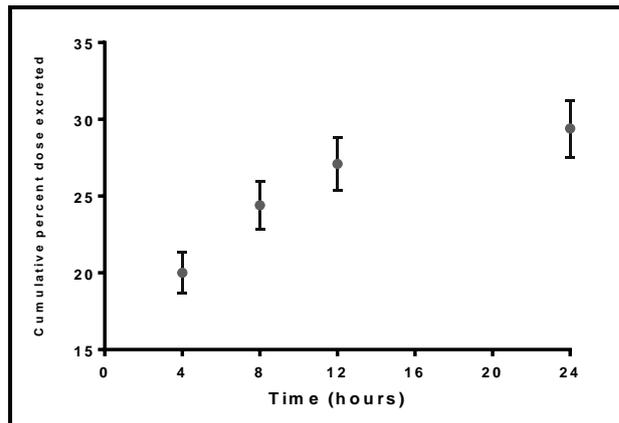


Fig. 5: Mean \pm SD values for cumulative percent dose of marbofloxacin excreted in the urine of 8 Lohi sheep following single intravenous administration at a dose of 2.5 mg/kg body weight

Discussion

In the current study, a specific, rapid and cost-effective HPLC method using UV/Vis detector for the estimation of marbofloxacin in plasma and urine was developed. Marbofloxacin possesses two ionizable groups in its structure and showed ionic properties at the specific range of pH. The use of tetraethyl ammonium bromide, an ion pairing reagent, significantly improved the separation of marbofloxacin from other components in plasma and urine. The composition of mobile phase, effluent flow rate, dimensions of the column and the type of detector greatly influence the results. So, the assay and its sensitivity must be taken into consideration when interpreting the data obtained in the course of any kinetic study.

The mean value of GFR in the current study was recorded as 1.29 ± 0.22 ml/min/kg. Our findings regarding GFR are higher compared to the previously documented values such as 1.0 ml/min/kg (Alvi *et al.*, 1985), 1.05 ± 0.06 ml/min/kg in ewes (Nawaz *et al.*, 1992), 1.13 ± 0.24 ml/min/kg (Nawaz *et al.*, 1992), 1.32 ± 0.35 ml/min/kg (Javed *et al.*, 2009) and 1.19 ml/min/kg (Afzal *et al.*, 1982) in Lohi sheep. Contrary to these results, much lower values (0.042 ± 0.005 ml/min/kg) of GFR were reported in local sheep (Javed *et al.*, 2005). GFR is an index of creatinine clearance. Creatinine is a waste product of muscle degradation which is continuously excreted by the kidneys via glomerular filtration process. The creatinine clearance depends upon the muscle mass of the animal, rate of muscle degradation (activity of the animal), rate of blood flow to the kidneys and the intrinsic activity of the kidneys. The variation in the GFR values might be due to one of the above said factors or because of the individual genetic variations among animals.

With the results of correlation analysis, it is evident that the marbofloxacin clearance strongly relates with the urinary flow rate and the drug plasma concentration. Urinary flow rate has positive relation to the marbofloxacin clearance which means that with the

increase in urine production the excretion of the drug also increases. Plasma concentration, on the other hand, has opposite relation to the marbofloxacin clearance in Lohi sheep.

Following absorption, fluoroquinolones show rapid and extensive tissue distribution due to hydrophilic nature and low (<50%) protein binding. In the current study, the extent of marbofloxacin binding to plasma proteins of Lohi sheep ($19.08 \pm 1.86\%$) was in accordance with the corresponding values of 19.1% for levofloxacin in buffalo calves (Dumka *et al.*, 2008) and 26% for danofloxacin in cattle (Giles *et al.*, 1991). The values of β_1 and K_β were 2.1×10^{-8} mol/g and 1.9×10^{-7} mol, respectively, in the current study, indicating weak and reversible binding of marbofloxacin to plasma proteins in Lohi sheep. The protein binding of a drug greatly influence the drug clearance as only the unbound and free drug can be eliminated from the kidneys. The fluoroquinolones are largely eliminated uncharged in the urine by glomerular filtration and active tubular secretion (Blum, 1992). Glomerular filtration is a unidirectional and size-selective process which allows passing only small molecular weight compounds up to 70000 Dalton (Christopher, 2012). Active tubular secretion is a carrier mediated transport mechanism which involves many drug transporters such as ABC efflux transporters (P-gp, MRP4 and MRP2), organic anion uptake transporter (OAT1 and OAT3) and organic cation uptake and efflux transporters (OCT2, OCTN and MATE). The high value (7.85) of marbofloxacin/creatinine clearance ratio suggests that the both mechanisms glomerular filtrations as well as active tubular secretion are involved in the marbofloxacin excretion from the body. The product of unbound fraction of marbofloxacin and urinary flow rate is higher than the creatinine clearance indicating the involvement of active tubular secretion in the renal handling of marbofloxacin. However, further detailed studies should be conducted in order to explore the exact excretory mechanism of marbofloxacin in animals.

In the current study, the urinary recovery of marbofloxacin was 29.4% after 24 h of drug administration. The maximum excretion rate appeared within 4 h in Lohi sheep and decreased continuously afterwards. However, the drug levels remained ≥ 5.0 $\mu\text{g/ml}$ in urine up to 24 h following IV administration at a dose of 2.5 mg/kg body weight in these animals. The mean concentration of marbofloxacin in urine remained many folds higher than the MIC range (0.015-0.50 $\mu\text{g/ml}$) for most microorganisms sensitive to the drug up to 24 h in Lohi sheep. Hence, it can be predicted that the use of marbofloxacin at a dose of 2.5 mg/kg in Lohi sheep might achieve successful bacterial killing in urinary tract infections caused by microorganisms having susceptibility ≤ 0.50 $\mu\text{g/ml}$ during 24 h of drug administration.

On the basis of the present study, it is concluded that the renal clearance of marbofloxacin is different in Lohi sheep when compared to its foreign counterparts. The drug is likely to be excreted by glomerular filtration process; however, other excretory mechanism may also

be involved. Further detailed studies at molecular level are required to explore the exact excretory mechanisms of fluoroquinolones in animals.

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