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Excretion of Levofloxacin into Saliva in Renal Failure Rat Model

[1]N.W. Sheikh, [1]A.S. Tripathi, [1]V. Chitra, [1]A. Choudhury and [2]S.P. Dewani
[1]SRM College of Pharmacy, Kathankulathur, Chennai, India
[2]Institute of Pharmaceutical Education and Research, Hinganghat Road, Wardha, India

Abstract: To clarify effects of renal failure on salivary distribution of Levofloxacin (LOFLX), a quinolone antibiotics, blood and saliva were collected from the double-step 5/6th-nephrectomized and sham-operated (control) rats after bolus i.v., administration of Levofloxacin (10 mg kg^{-1}). The concentrations of Levofloxacin in these samples were determined by high-performance liquid chromatography. Renal failure induced by the partial nephrectomy significantly elevated plasma levels and cumulative salivary excretion of Levofloxacin when compared to control rats. Total body clearance was significantly decreased by the renal failure, although salivary clearance of the partially nephrectomized rats was larger than that of the control. The saliva/plasma concentration ratios in the rats with renal failure were significantly greater than those in the control rats. The results of this study suggest that the salivary excretion of Levofloxacin is significantly increased by renal failure.

Key words: Levofloxacin, Renal failure rat model, therapeutic drug monitoring

INTRODUCTION

Levofloxacin is a synthetic fluoroquinolone antimicrobial agent with potent and broad activities against both gram-negative and gram-positive pathogens (Garrison, 2003) and is widely prescribed as the drug of choice to treat various infectious diseases. New quinolone antibiotics including LOFLX have been known to have severe adverse effects such as seizures and rhabdomyolysis. It has been reported that LOFLX undergoes a limited metabolism and is primarily excreted by kidney mainly as active drug, inactive metabolites (N-oxide and demethyl metabolites) represent less than 5% of the total dose (Albarellos *et al*., 2005). Its clearance is approximately 9 L h^{-1} and its half life is approximately 7 h in healthy subjects, in renal failure LOFLX clearance is reduced by approximately 75% and half life is prolong to 35 h (Czock *et al*., 2006). Additionally, many investigators have reported the pharmacokinetic interaction that gastrointestinal absorption of new quinolones is inhibited or reduced by antacids (Johnson *et al*., 1988). Therefore, LOFLX may be a quinolone dosage adjustment of which is required on the basis of the therapeutic drug monitoring.

Drug monitoring using the saliva offers a convenient and noninvasive alternative to blood analysis with particular advantages in geriatric and pediatric cases. For several drugs, it was reported that determination of saliva levels was successfully used for the therapeutic drug monitoring. It has been reported that the lipophilicity of the fluoroquinolones primarily determines the extent of salivary excretion (Langlois *et al*., 2005) and LOFLX has intermediate lipophilicity (Andersson and MacGowan, 2003) and has been demonstrated to show extensive distribution and good penetration into the extravascular compartment. It has been suggested that saliva might be substituted for plasma in therapeutic drug monitoring (Zuidema *et al*., 1996).

This study was conducted to clarify effects of renal failure on salivary distribution of Levofloxacin (LOFLX).

MATERIALS AND METHODS

Chemicals: LOFLX was kindly supplied by Micro Labs. Ltd. Pondicherry (India). All other reagents and solvents were commercially available and of analytical grade.

Animals: Ten to twelve-week-old male rats albino (wistar) weighing 300 to 350 g procured from King Institute, Guindy, Chennai (India) were used in this study. Animal Ethical Committee approved experimental protocol under guidelines of CPCSEA (662/02/C/CPSCEA, July 2002), New Delhi, India and Institutional Animal Ethical Committee (Approval Ref No. IAEC/27/2007). Animal were housed in a laboratory maintained a 12 h light-dark cycle and controlled room temperature (23±2°C) and relative humidity (50±10%). The animal received an appropriate diet (rat chow). A restricted feeding procedure was used

Corresponding Author: Naheed Waseem Sheikh, 28, Kohinoor Colony, Wani Road, Yavatmal-445 001 (M.S.), India
Tel: +917232241250, +919974756353, +919403937585

for at least 1 week before the study 15 g of feed was given per day at noon. This ensured overnight fasting for 14-16 h before surgical procedure. The project was conducted at SRM college of Pharmacy, SRM University Chennai, India, form 06/12/2007 to 06/03/2008.

Preparation of chronic renal failure model: The rat was anaesthetized with i.p. injection of pentobarbital (40-50 mg kg^{-1}) (Feinberg and Campbell, 1997). On the first day the left kidney was 2/3 nephrectomized and on the eight day the right kidney of the same rat was nephrectomized (Perez-Ruiz *et al*., 2006). The sham-operation (only incision and sutures on the abdomen) was performed on the controls.

The body weight, concentrations of plasma Creatinine and blood urea nitrogen in the sham-operated and 5/6th-nephrectomized rats were measured by using autoanalizer.

Pharmacokinetic study: The rat was anaesthetized with i.p. injection of pentobarbital (40-50 mg kg^{-1}). After tracheotomy and catheterization, the right jugular vein was cannulated with a silicon polymer tubing (I.D. 1.0 mm, O.D. 1.5 mm) for bolus administration of LOFLX and for collection of blood samples. Then the femoral vein was cannulated with a polyethylene tubing (PE-50; I.D. 0.58 mm, O.D. 0.965 mm) for constant-rate infusion of Pilocarpine hydrochloride (9 mg/h/kg) to stimulate salivation. Throughout the experiments, the body temperature of rats was maintained at 37.5°C using a heated pad.

The rat receive the constant-rate infusion of pilocarpine for 2 h to stabilize the salivation (Garcia-Olmos *et al*., 2003), the rats received a bolus i.v., injection of LOFLX at a dose of 10 mg kg^{-1}. Blood samples were collected by jugular vein (Thrivikraman *et al*., 2002) just before drug administration (about 200 il) and at designated times of 3, 5, 10, 20, 30, 40, 60, 80, 100, 120 and 140 min (about 100 μL) after administration and the plasma was immediately separated by centrifugation after heparinization. Saliva samples were separately collected at consecutive 10 or 20 min periods (0 to 10, 10 to 30, 30 to 50, 50 to 70, 70 to 90, 90 to 110, 110 to 130 and 130 to 150 min) after drug administration. Parotid and mendibular mixed saliva samples were collected by using dental cotton roll. The salivate is used to absorb the saliva into a dental cotton roll. Dental cotton roll after being socked with saliva, the container fits into a polystyrol tube that is then centrifuged for 3 min at about 1000 rpm. During centrifugation the saliva passes from the cotton roll into the lower part of the tube. The container is then taken out of the tube and the clear saliva is poured out of the tube. Cellular particles are retained at the bottom of the tube in a small sink compartment.

The pH of plasma and saliva: To determine the pH of plasma and saliva of the sham-operated and nephrectomized rats, the other rats received the surgical operation and saliva stimulation in the same manner as for the pharmacokinetic study. Saliva sample were separately collected under a liquid paraffin layer (about 0.15 mL) in a microtube during consecutive two 75 min periods from 2 h after the beginning of constant-rate infusion of pilocarpine. Blood samples were collected immediately before the saliva collection and midway through the collection period and the plasma were obtained by centrifugation after heparinization. Immediately after the collection of plasma and saliva, the pH of these samples was determined by a compact pH meter.

Sample extraction procedure: To 0.2 mL of heparinized plasma, 0.2 mL methanol was added. Plasma proteins were precipitated by shaking in an ultrasonic bath followed by centrifugation for 10 min at 2000 rpm. The supernatant was evaporate and reconstituted with mobile phase.

Drug analysis: Reverse phase high-pressure liquid chromatography (RP-HPLC) method was used for the calibration and determination of LOFLX in plasma and saliva, with column 5 μm; pump model LC-10 ATVP, Shmadzu, Japan; ultraviolet detector model SPD-10 A VP, Shimadzu, Japan was used to determine the concentrations of LOFLX in plasma and saliva of rat. (Santoro *et al*., 2006) the mobile phase was acetonitrile: water (80:20, v/v) stationary phase was Phenomenex Luna C_{18} Column (250×4.6 mm). The pH was adjusted to 3.5 by ortho phosphoric acid. The flow rate was 1.4 mL min^{-1} and the sample was determined at 296 nm.

Data analysis: Measured plasma concentration (C_p) time (t) data for LOFLX were analyzed on the basis of a two-compartment model expressed as the following equation:

$$C_p = A.\ e^{-\alpha t} + B.\ e^{-\beta t}$$

where, A, B, a, b are hybrid parameters. The nonlinear least-squares regression program WinNonlin was used for the regression analysis to obtain the hybrid parameters and secondary parameters, i.e., the elimination half-life ($t_{1/2\beta}$), total body clearance (CL_{tot}) and volume of distribution at the steady state (Vd_{ss}). The flow rate of

saliva was determined gravimetrically assuming the specific gravity to be approximately 1.0 .The S/P ratio of LOFLX was calculated as follows:

- The lag time for saliva collection was calculated from the salivary flow rate and the internal volume of saliva cannula
- The true midpoint of the saliva collection period was presumed from the lag time
- The plasma LOFLX concentration at the true midpoint was predicted by the two compartment model equation for the each rat
- The S/P ratio was expressed as the measured saliva concentration divided by the predicted plasma concentration

The salivary clearance (CL_{sal}) of LOFLX was calculated by multiplying the S/P ratio by the total salivary flow rate (per body weight) into which the measured flow rate of the single side was doubled, assuming that salivary LOFLX concentrations and salivary flow rates in the both sides would be equal.

Statistical analysis: The results were expressed as the Mean±SD for the indicated numbers of experiments. The significance of differences between the mean observations for two groups was determined using Student's t-test. Repeated measures analysis of variance was used to test for differences in the plasma concentration-time profiles between the treatments.

RESULTS

Levofloxacin assay: Calibration curves for LOFLX in the plasma and saliva were satisfactorily linear over the concentration ranges from 1 to 16 and 1 to 16 μg mL^{-1}, shown in the Table 1 and 2, respectively. The coefficients of variation for the assay were 2.15% at 2 μg mL^{-1} of the plasma concentration and 3.32% at 4 μg mL^{-1} of the saliva concentration. The respective regression equations for plasma and saliva were

$$y = 238950.2\,x + 5607.514 \quad (r = 0.999)$$

$$y = 276625.2x + 25097.29 \quad (r = 0.999)$$

where, y is the peak-area of the drug to the internal standard, x is the concentration in plasma or saliva (mg ml^{-1}) and r is the coefficient of correlation. The limits of determination were established at 1 μg mL^{-1} in plasma and at 1 μg mL^{-1} in saliva. Blank plasma and saliva samples did not interfere with the peaks for LOFLX.

Table 1: Linearity values for LOFLX in plasma by RP-HPLC Method

Concentration (μg mL^{-1})	Peak area
0	0
1	280900
2	460934
4	956672
8	1906891
16	3835704

Table 2: Linearity values for LOFLX in saliva by RP-HPLC method

Concentration (μg mL^{-1})	Peak area
0	0
1	288739
2	577478
4	1143582
8	2296183
16	4419982

Table 3: Body weight and biochemical data of sham-operated and nephrectomized rats

Measurement	Sham-operated[a]	Nephrectomized[a]
Body weight before treatment (g)	332.50±5.57	341.01±7.96
Body weight After treatment (g)	338.66±5.31	330.33±5.39
Plasma Creatinine (mg dL^{-1})*	0.44±0.039	0.85±0.074
Blood urea nitrogen (mg dL^{-1})*	35.40±7.31	135.33±15.38

[a]Data are expressed as the Mean±SD. *There are significant difference from sham-operated rats (student's t-test *$p<0.0001$)

Pathophysiological data: The body weight, concentrations of plasma Creatinine and blood urea nitrogen in the sham-operated and 5/6th-nephrectomized rats are summarized in Table 3. Plasma Creatinine levels were less than the detection limit of 0.5 mg dL^{-1} in sham-operated rats, whereas nephrectomized rats had higher Creatinine levels of about 0.85±0.074 mg dL^{-1}. Plasma urea nitrogen levels in the nephrectomized rats were about four times as high as those in the sham-operated rats.

Plasma and saliva pH: The pH of plasma obtained from the sham-operated and nephrectomized rats ranged from 7.2 to 7.7 throughout the experiment. Mixed saliva had the pH of 7.8 and 8.1, respectively, in both pretreatment groups. In the plasma and saliva pH, there was no difference between the sham-operated and nephrectomized groups and between the initial and latter halves of the sampling periods.

Plasma concentration-time profiles of LOFLX: In the nephrectomized rats, higher plasma LOFLX concentrations were observed when compared to the sham-operated rats shown in Table 4. In both groups, plasma concentrations of LOFLX were found to decline biexponentially with time.

Two-compartment model was the most adequate to describe the time courses on the basis of the minimum AIC estimation. The corresponding pharmacokinetic

Table 4: Geometric means/geometric SD (range) of LOFLX in plasma were tabulated against different time interval

Time (min)	Sham-operated[a] ----------(μg mL^{-1})----------	Nephrectomized[a]
3	7.24±0.0547	9.12±0.0865
5	6.60±0.0509	8.34±0.0789
10	5.62±0.0413	6.95±0.0659
20	3.98±0.0307	4.99±0.0468
30	3.31±0.0250	4.04±0.0377
40	2.69±0.0213	3.45±0.0343
60	2.29±0.0213	2.82±0.0258
80	1.94±0.0178	2.40±0.0236
100	1.69±0.0172	2.11±0.0194
120	1.54±0.0172	1.99±0.0175
140	1.41±0.0132	1.83±0.0167

Table 5: Pharmacokinetic parameters of LOFLX after i.v., bolus administration to sham operated and nephrectomized rats

Parameter[a]	Sham-operated[b]	Nephrectomized[b]
A (μg mL^{-1})	5.43±0.0891	7.01±0.1577
B (μg mL^{-1})	2.58±0.1050	3.03±0.0308
α (10^{-2} min^{-1})	5.68±0.1235	5.73±0.0468
β (10^{-3} min^{-1})	4.29±0.2131	3.73±0.1160
$T_{1/2\beta}$ (min)	161.98±8.2460	185.90±5.938
CL_{tot} (mL/min/kg)	12.51±0.2674	9.378±0.2836
V_{ss} (mL kg^{-1})	2922.10±99.517	2513.83±14.308

[a]A, B, α and β: Hybride parameters: $t_{1/2\beta}$: Elimination half life: C_{tot}: Total body clearance V_{ss}: distribution volume at steady state. [b]Data are expressed as the Mean±SD of six rats. There are difference two groups

Table 6: Cumulative excretion of LOFLX into saliva after i.v., bolus administration to sham-operated and nephrectomized rats

Time (min)	Sham-operated[a] ----------(μg mL^{-1})----------	Nephrectomized[a]
0-10	0.0260±0.0039	0.0294±0.0041
10-30	0.0360±0.0047	0.0472±0.0049
30-50	0.0443±0.0038	0.0593±0.0048
50-70	0.0520±0.0037	0.0690±0.0038
70-90	0.0602±0.0033	0.0781±0.0031
90-110	0.0667±0.0034	0.0854±0.0043
110-130	0.0715±0.0040	0.0914±0.0033
130-150	0.0768±0.0036	0.0969±0.0044

parameters of LOFLX are summarized in Table 5. The CL_{tot} was significantly decreased to about 25% by the partial nephrectomy and the $t_{1/2\beta}$ of the nephrectomized rats tended to be longer than that of sham-operated control. It was observed that V_{ss} of sham-operated was higher as compare to nephrectomized rats.

Salivary excretion: In sham operated rats, the cumulative salivary excretion of LOFLX up to 150 min after administration was 0.0768% of the dose Table 6. The nephrectomy induces a significant increase in the cumulative salivary excretion of LOFLX in saliva. The salivary flow rates and CL_{sal} of LOFLX were calculated from the mean observations of three collection periods from 90 to 150 min after administration and were compared between two groups of the rats. The salivary flow rate in the sham-operated rats was 18.26±1.2 μL/min/kg. The nephrectomy tended to increase the flow rates of saliva to 22.19±1.5 μL/min/kg, respectively, although there was significant difference between sham-operated and nephrectomized rats.

Table 7: Measured and predicted S/P ratio of LOFLX after bolus i.v., administration to sham-operated and nephrectomized rats

	Measured[a]		Predicted[b]	
	Sham-operated	Nephrectomized	Sham-operated	Nephrectomized
Saliva	0.99±0.045	1.25±0.026	1.21	1.36

[a]Data expressed as the Mean±SD. The number of rats is indicated in the parentheses. They are based on the mean of the values obtained from 90 to 150 min after drug administration in individual rats. There are non significant differences from sham-operated rats (student's t-test: $p>0.05$). [b]They are predicted with the equation: S/P ratio = $[(1+10^{\,pHs-pKa2})\,f_p] / [(1+10^{\,pHp-pKa2})\,f_s]$

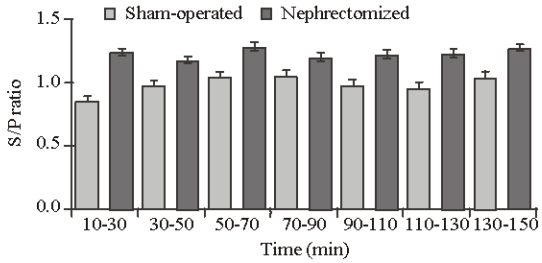

Fig. 1: S/P Ratio of LOFLX after bolus administration to sham-operated and nephrectomized rats. Each point represents the Mean and SD of six rats. There are a significant difference of cumulative excretion between sham-operated and nephrectomized group (student's t-test $p<0.0001$)

The CL_{sal} of LOFLX was 27.73 and 18.07 μL/min/kg for nephrectomized and sham operated rats saliva. The S/P ratios of LOFLX for each saliva are shown in Fig. 1. In each group, the S/P ratios were nearly constant during the periods, which corresponded to the elimination phase of the plasma concentration-time profile. The S/P ratios in the nephrectomized rats were slightly higher than those in the sham-operated control during 70 to 150 min. after administration.

Prediction of S/P ratio by Matin's equation: In as much as LOFLX is weakly acidic, the following equation may predict the saliva to plasma concentration ratio.

$$\frac{C_s}{C_p} = \frac{1+10\,(pK_a\text{-}pH_s)\times f_p}{1+10\,(pK_a - pH_p)\times f_s}$$

where, pH_s and pH_p are pH values of saliva and plasma, respectively; f_s and f_p are free fraction of total drug concentration in saliva and plasma, respectively. Table 7 summarized the observed and predicted S/P ratio for sham-operated and nephrectomized rats in saliva samples according to the above equation.

DISCUSSION

The use of saliva instead of blood for pharmacokinetic investigations has obvious practical advantages. It is a painless, non-invasive procedure, hence suitable for the collection of multiple specimens. Variable results have been reported for the ratio of saliva to serum concentrations (Edelstein *et al*., 1996).

The present study shows that several pharmacokinetic parameters can be calculated using salivary concentrations of LOFLX as can be done with plasma. The estimation of LOFLX levels in saliva seems to be useful in therapeutic drug monitoring and pharmacokinetic studies.

Lot of methods are available for producing renal failure in experimental animals, which include the administration of various nephrotoxic agents such as uranyl nitrate, mercurials, cisplatin,and ureteral ligation, partial nephrectomy. Although the chemical methods such as uranyl nitrate injection are simple and reliable (Giacomini *et al*., 1981) at the nephrotoxic agents could also damage salivary glands. Therefore, in this experiment, the double-step 5/6th-nephrectomy was used to induce the experimental renal failure. This surgical method can damage only the kidneys, so that it is possible to investigate the effects of renal failure per se on salivary excretion of the drug.

Plasma creatinine, blood urea nitrogen levels were remarkably raised by the partial nephrectomy, indicating that severe renal failure was produced. The saliva flow rate in the rats with renal failure tended to be larger than those of the control, impairment of saliva secretion was not induced by renal failure in rats. The elevated secretion of saliva may be in compensation for decreased urine production.

Based on the salivary levels, the present study compares the pharmacokinetic parameters of LOFLX in sham operated rat and in 5/6th nephrectomized rat model. The pharmacokinetic parameters of LOFLX such as $t_{½}$, CL_{Tot} and Vd_{ss} does change when given in 5/6th nephrectomized rat model. Suggest that the pharmacokinetics of LOFLX get affected or altered when administered chronic renal failure rat model.

Salivary excretion and S/P ratio of LOFLX in saliva were significantly higher than those in control rats. Generally, it is known that the S/P ratio of weakly acidic or basic drugs depends on the salivary pH. As for quinolone antibiotics, it was reported that salivary penetration of Levofloxacin was pH-dependent and higher saliva levels of Levofloxacin were found in the more acidic samples. Therefore, in this study, a theoretical S/P ratio of LOFLX were calculated according to the pH partition theory and compared to the measured ratios. Furthermore, the measured S/P ratios were less than the values predicted according to the pH-partition theory where the zwitterionic species were assumed to predominantly penetrate into salivary glands by passive diffusion. Thus, LOFLX distribution into saliva could not be apparently explained by the pH-partition theory itself. In this theory, it is assumed that a nonionized (or zwitterionized) species is sufficiently lipophilic and rapidly diffuse across the membrane. If a zwitterionic form of LOFLX was much less lipophilic than the uncharged form, lower S/P ratios compared with the theoretical values would not be unexpected. Another explanation for the discrepancy between measured and theoretical S/P ratios can be discussed. Passive diffusion through the membrane may not be only the mechanism for LOFLX penetration into saliva. Possible active transport system, which could pump out this quinolone from saliva to the circulation, may operate.

It has been known that there are specific transport systems in the salivary glands (Novak, 1990). The transport systems could actively carry not only endogenous substrates but also exogenous materials including various drugs through the salivary gland epithelium membranes. Recently, it has been reported that the specific transport systems for quinolone antibacterial agents including LOFLX exist in the epithelium membranes of rat choroid plexus (Soriano and Rodriguez-Cerrato, 2002). Since, salivary glands also have the epithelial membrane, which is morphologically similar to choroidal and renal tubular epithelium, it is likely that a specific system for LOFLX may function in the salivary glands and LOFLX may be reabsorbed from saliva via this system. Relatively low salivary distribution of this quinolone in rats may be due to this putative efflux mechanism, which could transport LOFLX from saliva to blood.

Interesting results were obtained concerning the effect of nephrectomy on salivary excretion of LOFLX. The measured S/P ratios in the group of renal failure were greater than the control saliva, suggesting that LOFLX distribution between the blood and saliva may be altered in the rats with renal failure (Tsaganosa *et al*., 2008). In contrast, it was reported that saliva and plasma levels of procainamide, a basic drug, were higher in renal-impaired rats by means of two-step subtotal nephrectomy but the S/P ratio was unchanged in comparison to the controls. Thus, renal failure may cause different changes in the salivary distribution of different types of drugs (Tomas *et al*., 2008).

A few possibilities could be discussed on the mechanisms for enhanced salivary distribution of LOFLX in renal failure. In salivary gland, acinus cells form the barrier for drug translocation between blood and saliva. Renal insufficiency may induce destruction of the blood-saliva barrier leading to increase of drug distribution into saliva. In fact, detected amyloid-like

fibrils of a salivary gland in patients with renal insufficiency. Another possibility is related with the putative-specific transport in salivary glands. The activity of the possible efflux system for LOFLX may be inhibited by uremic toxins which increase in renal failure, resulting in accumulation of LOFLX in saliva. Alternatively, the possibility of the secondary change in the S/P ratio by elevated LOFLX levels in plasma and saliva induced by renal failure could be considered. Salivary distribution of LOFLX might be operated by a concentration-dependent system affected by elevation of the plasma and/or saliva concentration of the drug (Kinashi *et al*., 1989).

The parallel bi-exponential decline of salivary LOFLX level with the plasma gave almost the same values for both α and β as those in the plasma level sample, suggesting that the salivary drug level may be substituted for the plasma level if consistent S/P ratio is obtained over a broad concentration range. Since the LOFLX level in saliva was directly proportional to that in plasma, the plasma pharmacokinetics could be predicted almost completely.

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