Evaluation of Saliva as a Potential Alternative Sampling Matrix for Therapeutic Drug Monitoring of Levofloxacin in Patients with Multidrug-Resistant Tuberculosis

Samiksha Ghimire,^a Bhagwan Maharjan,^b Erwin M. Jongedijk,^a Jos G. W. Kosterink,^a Gokarna R. Ghimire,^c Daan J. Touw,^{a,d} Tjip S. van der Werf,^e Bhabana Shrestha,^b Jan-Willem C. Alffenaar^{a,f}

^aUniversity of Groningen, University Medical Center Groningen, Department of Clinical Pharmacy and Pharmacology, Groningen, The Netherlands

^bGerman Nepal TB Project, Nepal Anti-Tuberculosis Association, Kathmandu, Nepal

^cGovernment of Nepal, Ministry of Health and Population, Department of Health Services, National Tuberculosis Center, Kathmandu, Nepal

^dUniversity of Groningen, Groningen Research Institute of Pharmacy, Department of Pharmacokinetics, Toxicology and Targeting, Groningen, The Netherlands

^eUniversity of Groningen, University Medical Center Groningen, Infectious Diseases Service and Tuberculosis Unit, Groningen, The Netherlands

^fUniversity of Sydney, Faculty of Medicine and Health, School of Pharmacy and Westmead Hospital, Sydney, Australia

ABSTRACT Saliva may be a useful alternative matrix for monitoring levofloxacin concentrations in multidrug-resistant tuberculosis (MDR-TB) patients. The objectives of this study were (i) to evaluate the correlation between plasma and salivary levofloxacin (Lfx) concentrations in MDR-TB patients and (ii) to gauge the possibility of using saliva as an alternative sampling matrix for therapeutic drug monitoring of Lfx in areas where TB is endemic. This was a prospective pharmacokinetic study that enrolled MDR-TB patients receiving levofloxacin (750- to 1,000-mg once-daily dosing) under standardized treatment regimen in Nepal. Paired blood and saliva samples were collected at steady state. Lfx concentrations were quantified using liquid chromatography-tandem mass spectrometry. Pharmacokinetic parameters were calculated using noncompartmental kinetics. Lfx drug exposures were evaluated in 23 MDR-TB patients. During the first month, the median (interquartile range [IQR]) areas under the concentration-time curve from 0 to 24 h (AUC_{0-24}) were 67.09 (53.93 to 98.37) mg · h/liter in saliva and 99.91 (76.80 to 129.70) mg · h/liter in plasma, and the saliva plasma (S/P) ratio was 0.69 (0.53 to 0.99). Similarly, during the second month, the median (IQR) AUC_{0-24} were 75.63 (61.45 to 125.5) mg · h/liter in saliva and 102.7 (84.46 to 131.9) mg · h/liter in plasma, with an S/P ratio of 0.73 (0.66 to 1.18). Furthermore, large inter- and intraindividual variabilities in Lfx concentrations were observed. This study could not demonstrate a strong correlation between plasma and saliva Lfx levels. Despite a good Lfx penetration in saliva, the variability in individual saliva-to-plasma ratios limits the use of saliva as a valid substitute for plasma. Nevertheless, saliva could be useful in semiquantitatively predicting Lfx plasma levels. (This study has been registered at ClinicalTrials.gov under identifier NCT03000517.)

KEYWORDS levofloxacin, pharmacokinetics, plasma, saliva, tuberculosis

Levofloxacin (Lfx) belongs to the group A fluoroquinolones (FQs) for treating multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least isoniazid and rifampin (1). This class of drug is used throughout the course of treatment in the new, shorter 9-month regimen; in the longer 24-month MDR-TB regimen; and additionally in the 6-month regimen for rifampin susceptible, isoniazid mono-resistant TB (2). Lfx and moxifloxacin have been used interchangeably in the longer regimen; however, in developing countries Lfx is preferred due to affordability, availability, a

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Address correspondence to Jan-Willem C. Alffenaar, j.w.c.alffenaar@umcg.nl.

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better safety profile, and fewer drug interactions with other medications (3, 4). Acquired FQ resistance during standard treatment resulting in poor outcomes shown in a prospective observational cohort study is of serious concern (5). An earlier study by the same group showed that 11.2% (79/832) of MDR-TB patients developed FQ resistance without any baseline resistance, potentially due to subtherapeutic systemic concentrations of drugs achieved (6, 7). Similarly, other pharmacokinetic studies on Lfx in MDR-TB patients found considerable pharmacokinetic variability among individuals, with at least 25% of the patients not attaining desired plasma concentration and area under the concentration-time curve from 0 to 24 h (AUC_{0-24}) (3, 4, 8, 9). It is clear that Lfx concentrations do not always reach the desired concentrations while administered in a standard dose. Therefore, measuring Lfx concentrations in plasma or other alternative matrices (saliva and dried blood spots) could help clinicians make informed dosing decisions, especially now, as the TB treatment marches toward individualization, therapeutic drug monitoring (TDM) using saliva sampling might become a game changer in TB treatment due to specific advantages over plasma sampling, in low-resource settings (10, 11). The efficacy of Lfx is predicted by AUC_{0-24} and MIC (12). Given as a monotherapy, the hollow-fiber infection model on TB recently established a Lfx target of 146 for maximum bacterial kill (80% effective concentration) and 360 for the prevention of acquired drug resistance (12). Therefore, a plasma AUC_{0-24} above 75 (if the MIC is 0.5 mg/liter) or 146 (if the MIC is 1 mg/liter) will be needed to attain the optimal target exposure for efficacy. With standard 750 to 1,000 mg once daily dose, desired median peak concentration (C_{max}) was 8 to 13 mg/liter, while the median time to reach C_{max} (t_{max}) was 1 to 2 h and the median half-life ($t_{1/2}$) was 6 to 8 h (13–15). Lfx demonstrated good penetration in extravascular body sites such as cerebrospinal fluid and cavitary lesions, due to rapid absorption and high volume of distribution (16, 17). Sasaki et al. compared the Lfx levels in saliva and sera from eight healthy male volunteers after the administration of a 100-mg single dose. The study reported a mean saliva/serum Lfx AUC ratios of 0.69 in a fasting state and 0.56 in a nonfasting state, indicating that saliva Lfx concentration could be useful for TDM (18). To date, however, studies comparing Lfx concentrations in plasma and saliva of MDR-TB patients have not been published. Saliva could be a useful alternative in predicting Lfx concentrations from plasma since sampling is noninvasive, is fast, requires less rigid storage conditions, can be easily transported, and is more suitable in children (19).

Therefore, the aims of the present study were (i) to evaluate the correlation between plasma and salivary Lfx concentrations in MDR-TB patients and (ii) to gauge the possibility of using saliva as an alternative sampling matrix for TDM of Lfx in areas of TB endemicity.

RESULTS

Study subjects. Twenty-three MDR-TB patients were included in the study and demographic and baseline clinical characteristics are shown in Table 1. In our study, 70% (16/23) of the subjects were male. The median age was 32 (interquartile range [IQR], 28 to 47 years), and the body weight was 48 (IQR, 41 to 55 kg) with a body mass index (BMI) of 18 (IQR, 16 to 19 kg/m²). Based on the BMI, 65% (15/23) of the patients were underweight, as a result once-daily 750- to 1,000-mg Lfx dosing resulted in mg/kg doses of 17.14 (15.38 to 19.23). All 23 (100%) patients completed the first pharmacokinetic (PK) sampling (days 15 to 30). However, during the second month, 4 (13.1%) patients failed to participate in PK sampling. One patient was transferred out, while two patients were shifted to a pre-extensively drug-resistant category, whereas the remaining patient participated only in saliva sampling.

Lfx PK. The steady-state Lfx PK parameters are mentioned in Table 2. During the first month, the median area under the concentration-time curve (AUC_{0-24}) was 67.09 (IQR, 53.93 to 98.37) mg · h/liter in saliva and 99.91 (IQR, 76.80 to 129.70) mg · h/liter in plasma, and the saliva/plasma (S/P) ratio was 0.69 (IQR, 0.53 to 0.99). Moreover, the median C_{max} was 7.03 (IQR, 5.61 to 9.02) mg/liter in saliva and 10.35 (9.10 to 11.44) mg/liter in plasma with an S/P ratio of 0.68 (IQR, 0.53 to 0.97). A moderate positive

TABLE 1 Baseline characteristics of all included patients

Patient characteristics	Value, no. (%) or median (IQR)
Demographic data (<i>n</i> = 23)	
No. male	16 (69.56)
Age (yr)	32 (28–47)
Body wt (kg)	48 (41–55)
Length (cm)	165 (162–175)
BMI (kg/m ²)	17.96 (16.23–18.83)
Underweight (<18.5 kg/m ²)	15 (65.22)
Normal (18.5–25.0 kg/m ²)	8 (34.78)
Comorbidities	
Diabetes mellitus	2 (8.69)
HIV	0
Dose (mg/kg)	
Lfx	17.14 (15.38–19.23)
Renal function, baseline	
Creatinine (μmol/liter)	70.72 (61.88–79.56)
Urea (mg/dl)	19 (15–23)
Sodium (mmol/liter)	140 (134–144)
Potassium (mmol/liter)	4.12 (3.83–4.4)

correlation ($r_s = 0.50$; $P = 0.016$) was demonstrated between the saliva and plasma AUC_{0-24} values. Similarly, during the second month, the median AUC_{0-24} and C_{max} were 75.63 (IQR, 61.45 to 125.5) mg · h/liter and 8.30 (IQR, 6.56 to 12.03) mg/liter in saliva and 102.7 (IQR, 84.46 to 131.9) mg · h/liter and 10.96 (IQR, 9.34 to 11.58) mg/liter in plasma. The median AUC_{0-24} S/P ratio was 0.734 (IQR, 0.66 to 1.18). This time, the saliva and plasma AUC_{0-24} values showed a strong positive linear relationship ($r_s = 0.754$; $P = 0.0001$) compared to the first month. Assuming a Lfx plasma protein binding of 24%, the median free plasma $fAUC_{0-24}$ was 75.93 (IQR, 58.37 to 98.57) mg · h/liter in the first month and 78.05 (IQR, 64.19 to 100.24) mg · h/liter in the second month of treatment. The median S/P ratios were 0.96 (IQR, 0.95 to 1.25) and 0.88 (IQR, 0.92 to 0.99), respectively. The unbound Lfx $fAUC_{0-24}$ in plasma reflected its salivary AUC_{0-24} closely, with an S/P ratio close to 1. The Lfx concentration-time curves for both plasma and saliva are shown in Fig. 1.

Furthermore, a trend toward moderately positive correlation ($r_s = 0.379$; $P = 0.021$) was observed when Lfx C_{min} in saliva was evaluated to predict its AUC_{0-24} in plasma ($r = 0.38$; estimated linear regression equation). When the saliva C_{min} was <2 mg/liter, a proportion of patients had a plasma AUC_{0-24} below the desired level, 146, given the MIC was 1 mg/liter (12).

Passing Bablok regression analysis was used to evaluate the agreement between plasma and saliva Lfx concentrations. Figure 2 shows a fitted Passing-Bablok regression that revealed a linear relationship and was close to the line of identity ($x = y$), with an estimated slope 95% confidence interval (CI) of 1 (−2.11 to 2.57) for the first month and 1.81 (−0.51 to 3.92) for the second month. Similarly, the intercepts were −1.85 (−9.81 to 16.42) and −7.17 (−21.26 to 0.95), respectively. In both months, 95% CI range included 1 for slope and 0 value for intercept, thereby satisfying the condition for line of identity.

The interindividual variability was assessed in 208 Lfx measurements in plasma and 195 measurements in saliva for the samples at 0, 1, 2, 4, and 8 h. We found a large interindividual variability in Lfx concentrations. Furthermore, the intraindividual variability was evaluated for the same patient based on the Lfx concentrations in plasma and saliva between the first and second months of treatment. The median intraindividual variability (CV_{intra}) was 8.77 (IQR, 3.56 to 24.90%) in plasma and 24.25 (IQR, 12.20 to 34.65%) in saliva for 19/23 patients. In our study, the intraindividual variability was lower than the interindividual variability. Table 3 shows inter- and intraindividual coefficients of variation (CVs) for Lfx. The salivary pH ranged from 4.5 to 8.0 for different

TABLE 2 Steady-state pharmacokinetic parameters for Lfx^a

Parameter	Plasma		Saliva		S/P ratio		P value (I and II)	
	I (n = 23)	II (n = 19)	I (n = 23)	II (n = 20)	I	II	Plasma	Saliva
AUC ₀₋₂₄ (mg · h/liter)	99.91 (76.80–129.70)	102.7 (84.46–131.90)	67.09 (53.93–98.37)	75.63 (61.45–125.5)	0.69 (0.53–0.99)	0.74 (0.59–0.93)	1.00	0.05
fAUC ₀₋₂₄ (mg · h/liter)	75.93 (58.37–98.57)	78.05 (64.19–100.24)	7.03 (5.61–9.02)	8.30 (6.56–12.03)	0.88 (0.92–0.99)	0.96 (0.95–1.25)	0.17	
C _{max} (mg/liter)	10.35 (9.10–11.44)	10.96 (9.34–11.58)	2 (1.3–4)	2(1.04–3.36)	0.68 (0.53–0.97)	0.73 (0.66–1.18)	0.72	0.07
t _{max} (h)	2 (1.08–4)	2 (1–2.06)	2 (1.3–4)	2(1.04–3.36)			0.34	0.23
CL/F (liters/h)	6.75 (4.72–9.46)	7.94 (5.09–9.34)	9.58(6.74–12.33)	8.99 (5.92–11.80)			0.93	0.52
V/F (liters)	87.9 (72.54–106.40)	88.84 (55.73–101.2)	124.3 (111.45–157.30)	125.60 (83.04–158.25)			0.13	0.87
t _{1/2el}	8.77 (6.50–10.71)	7.86 (6.32–10.11)	8.58 (7.97–10.36)	8.47 (6.23–14.02)			0.94	0.96
k _{el} (/h)	0.08 (0.06–0.11)	0.08 (0.07–0.08)	0.10 (0.07–0.12)	0.08 (0.05–0.11)			0.94	0.96

^aData for months I and II are presented as medians (IQRs) where applicable. Abbreviations: AUC₀₋₂₄, area under the concentration-time curve from 0 to 24 h; fAUC₀₋₂₄, free Lfx AUC₀₋₂₄, assuming a plasma protein binding of 24%; C_{max}, maximum concentration of drug; t_{max}, time at which C_{max} occurred; CL/F, apparent total body clearance; V/F, apparent volume of distribution; t_{1/2el}, elimination half-life; k_{el}, elimination rate constant.

FIG 1 Lfx plasma and saliva concentration-time curves (means $\pm$ the standard errors of the mean).

individuals, with a mean value of 5.78 in the first month and 5.96 in the second month. Lfx saliva and plasma ratios as a function of salivary pH are plotted together (Fig. 3).

DISCUSSION

The presence of Lfx in MDR-TB regimen has been associated with greater treatment success and reduced death (20). Despite this dominant position as a second-line TB drug, many clinical trials have demonstrated inadequate Lfx concentrations in the plasma of MDR-TB patients that prevent the drug from achieving its maximum efficacy (4, 8, 9). The measurement of drug concentrations in the plasma of MDR-TB patients and dose adjustments thereafter have contributed positively to MDR-TB treatment outcomes (21). However, only a few TB treatment centers worldwide have adopted TDM. Officially, the importance of TDM in the management of subgroups of patients with drug-susceptible tuberculosis was first introduced in the clinical practical guide-

FIG 2 Passing-Bablok regression analysis of mean Lfx concentrations (at 0, 1, 2, 4, and 8 h) in plasma and saliva for 2 months. The bold solid line represents the Passing-Bablok fitted line, whereas the solid lighter line is the line of identity. The dashed lines indicate the 95% CI, r is the Spearman's rank correlation, and N is the number of paired mean plasma and saliva concentrations.

TABLE 3 Interindividual and intraindividual variabilities of Lfx

Time (h)	Interindividual variability ($n = 23$) ^a			
	Mean plasma concn (mg/liter), SD; %CV _{inter}		Mean saliva concn, SD; %CV _{inter}	
	I	II	I	II
0	1.70, 1.14; 67.29	1.63, 1.06; 65.39	1.32, 1.02; 77.02	1.70, 1.60; 94.29
1	8.26, 2.47; 41.98	7.23, 3.65; 50.43	5.58, 2.88; 51.58	5.63, 4.34; 77.02
2	8.42, 2.36; 27.98	9.91, 1.90; 19.13	5.56, 2.70; 48.53	8.46, 3.80; 44.90
4	7.61, 2.04; 25.85	7.84, 1.92; 24.43	5.09, 3.10; 61.02	6.53, 5.28; 80.80
8	6.40, 3.72; 58.11	6.14, 3.37; 54.89	4.05, 2.31; 56.91	4.85, 3.01; 61.97

^aValues for months I and II are expressed as follows: "mean concn, standard deviation; %CV_{inter}," where "%CV_{inter}" is the percent interindividual coefficient of variation. The median %CV_{intra} (IQR) values for plasma and saliva were 8.77 (3.65 to 24.90) ($n = 19$) and 24.25 (12.20 to 34.65) ($n = 20$), respectively.

lines by the American Thoracic Society, the Centers for Disease Control and Prevention, and the Infectious Diseases Society of America and was endorsed by the European Respiratory Society and the U.S. National Tuberculosis Controllers association (22). Among many logistic and financial challenges that have hindered TDM implementation, one problem is that venous sampling does not always have enough leverage in low-resource TB-endemic settings, mainly due to the invasive nature of sampling, the need for skilled personnel for venipuncture, potential infectious hazards, cooling requirements for transportation and storage, and high costs (11). In this scenario, the use of alternative and stress-free sampling matrixes such as saliva could imprint TDM strategy in national TB treatment guidelines. Therefore, in this first study of salivary penetration of Lfx in MDR-TB patients, we evaluated saliva's potential as an alternative sampling matrix and explored whether it can quantitatively reflect plasma concentrations for TDM-guided dosing. Overall, the salivary and plasma concentration-time profiles agreed well for different patients characterized by higher Lfx concentrations in plasma than in saliva, except in 21% (5/23) of patients who had higher salivary concentrations. The amount of Lfx present in saliva is representative of its free fraction in plasma that is able to passively diffuse to saliva, which happens almost instantaneously due to a concentration gradient (23). Given Lfx's variable degree of protein binding (24 to 40%) in different individuals, we found large interindividual variation in salivary concentrations (20). The results obtained from plasma samples were more homogeneous and consistent with recently published studies on MDR-TB patients by van't Bovenind-Vrubleuskaya et al. and Peloquin et al., with similar median observed AUC_{0-24} and C_{max} values (4, 15). In theory, several factors could explain the high interindividual variability in saliva, such as salivary pH in combination with drug pKa, salivary flow rate, and mechanism of drug transport (passive or active) (23, 24). The degree of ionization in different compartments is generally explained by pH of the

FIG 3 Lfx saliva-plasma ratios at different time points (0, 1, 2, 4, and 8 h) and salivary pH values at the first (I) and second (II) months of treatment.

FIG 4 Lfx C_{min} in saliva to predict plasma AUC_{0-24} . The vertical line at 2 mg/liter is the C_{min} cutoff.

compartments and the pKa of the drug. For example, lipid-soluble nonionized drugs that are not extensively bound to plasma proteins can easily transfer across the phospholipid bilayer of salivary cell membranes compared to ionized hydrophilic ones, which tend to be retained in plasma (24, 25). The pKa values for Lfx are 5.35 (strongest acidic) and 6.72 (strongest basic) and a saliva pH range was 4.5 to 8 (23). In patients with high salivary Lfx levels, it could be hypothesized that higher salivary concentrations could be the function of acidic salivary pH and basic drug pKa values that permitted swift transfer of Lfx from plasma to saliva and ionization thereafter. However, due to the unavailability of actual drug pKa data and unbound Lfx fraction in plasma for individual patients, we could not determine whether salivary pH alone explained the variabilities in salivary Lfx concentrations. In addition, the patient hydration state is thought to influence parotid salivary flow rates and in turn saliva-derived drug concentrations. Since saliva mainly constitutes water (97 to 99.5%) originating from plasma by acinar cells, it is hypothesized that decrease in water volume due to dehydration would result in the loss of salivary production (26). Ship and Fischer reported that dehydration significantly decreases the salivary output (27) but could not establish a strong correlation between biological markers of hydration (hematocrit, hemoglobin, serum sodium, plasma protein, creatinine, serum, and urine osmolality) and salivary output in their study (28). Therefore, influence of hydration/dehydration status on salivary Lfx concentrations needs to be studied. Furthermore, the presence of active transport channels might have contributed to high salivary concentrations, which have been studied for some peptides such as insulin but not for Lfx, and needs to be validated (25).

A noteworthy finding of our study was that Lfx in saliva does not accurately predict its plasma levels due to variable S/P ratios at different months of treatment and a large interindividual variability in Lfx saliva concentration %CV of 44.90 and 94.29% (minimum and maximum).

This observation is not surprising as anti-TB drugs (levofloxacin, moxifloxacin, isoniazid, rifampin, and linezolid, among others) exhibit high rates of PK variability even in plasma. Moreover, alternative matrices for TDM, such as dried blood spots and saliva, rarely match the precision of plasma-based assessments. Despite the limitations, the potential utility of saliva in semiquantitative testing remains strong. Patients with an Lfx C_{min} below 2 mg/liter in saliva were at the risk of a lower plasma AUC_{0-24} (Fig. 4). These patients are likely to benefit from semiquantitative saliva-based TDM in resource-limited settings. However, this simple and noninvasive saliva-based TDM may present a few challenges. First, the sampling procedure using a salivette (Sarstedt, Nümbrecht, Germany, catalog no. 50-809-199) introduces variability in recovery depending on the

type of cotton rolls used (plain cotton swab, cotton swab impregnated with citric acid, and synthetic cotton swab). We found that around 30% of Lfx was sorbed to plain cotton rolls used for collection of saliva samples. Therefore, the saliva sample collection procedure should be standardized and well controlled. The salivette technique further requires centrifugation for recovery of collected saliva. Alternatively, saliva samples could be collected by compressing the cotton roll in a syringe equipped with membrane filter (29). Second, it will not be feasible to analyze Lfx levels in saliva by advanced liquid chromatography-tandem mass spectrometry (LC-MS/MS) in resource-limited settings. It has been suggested that patients at risk for low FQ exposure can be distinguished from those with a normal or high exposure by a semiquantitative point-of-care test such as spectrophotometer/thin-layer chromatography at a local level (30). Early preselection using semiquantitative testing will act as a gatekeeper, only selecting patients at risk to offer TDM with an expensive high-performance liquid chromatography technique at the regional level, thereby optimally allocating resources from already-depleted TB programs (10, 30). Therefore, the development of a simplified, affordable, point-of-care tool for the determination of Lfx levels in saliva should be a priority. Third, the thermal instability of anti-TB drugs and the need for refrigeration and cooling conditions for transportation might be an issue. We recently investigated the impact of high-temperature exposure on the stability of Lfx and found that it was stable at 50°C for 10 days. This is advantageous, since it precludes the cooling requirements for transportation of samples to the laboratories for TDM. Preferably, in remote settings, dried blood spot sampling could be a feasible option due to longer stability at room temperature and transportation option by regular mail but still requires advanced LC-MS/MS for analysis. Another attraction in the field of alternative sampling could be dried saliva spots, but this requires sensitive, high-cost equipment, analytical method development and validation, and long-term stability testing at higher temperatures.

In this study, we could not use the Bland-Altman method for graphical representation of mean and 95% CI limits of agreement between Lfx concentrations in plasma and saliva. The one-sample *t* test showed that the log differences between saliva and plasma concentrations were significantly different ($P < 0.05$) from 0, which violates one of the assumptions of Bland-Altman analysis.

There were some limitations in our study. First, the sample size of 23 was rather small to explain the observed high Lfx inter- and inpatient variability in saliva compared to plasma. To explain this effect in saliva, studies with a sample size that ensures a statistical power of $>80\%$ will be needed. Second, different predictors of salivary Lfx concentrations, such as salivary flow rate, were not studied. Despite these limitations, salivary Lfx concentrations could contribute as a valuable semiquantitative preselection tool to identify patient subgroups eligible for TDM using dried blood spots. Patients with an Lfx $C_{\min}$ below 2 mg/liter in saliva could benefit from TDM due to the risk of a lower plasma AUC_{0-24} .

In conclusion, we could not demonstrate any significant relationship between plasma and saliva Lfx levels. Although Lfx penetrated in saliva, the variability in individual saliva/plasma ratios limits the use of saliva as a valid substitute for plasma. Despite the limitations, our data suggest that the potential utility of saliva in semiquantitative testing remains strong. Patients with an Lfx $C_{\min}$ below 2 mg/liter in saliva are likely to benefit from semiquantitative saliva based TDM in resource-limited settings.

MATERIALS AND METHODS

Patients and design. Study participants were MDR-TB patients undergoing treatment at German Nepal Tuberculosis Project (GENETUP), Nepal. This was a prospective pharmacokinetic study that enrolled patients on treatment from 25 May 2016 to 27 October 2017, with signed informed consents. The study protocol was approved by the Ethical Review Board of Nepal Health Research Council, Kathmandu, Nepal (115/2016), and is registered at clinicaltrials.gov (identifier number NCT03000517). Patients (≥ 18 years) with newly diagnosed or previously treated MDR-TB (based on genotypic susceptibility testing to rifampin by GeneXpert and culture) receiving Lfx as a part of their MDR-TB regimen were eligible for inclusion. Subjects were excluded if they had neurologic or severe extrapulmonary manifestations of TB; had a body weight <35 kg; were on medications for the treatment of renal disorders; were breast

feeding or pregnant; or were being treated with aluminum- and magnesium-containing antacids or ferrous sulfate, cimetidine, probenecid, theophylline, warfarin, zidovudine, digoxin, or cyclosporine due to potential drug-drug interactions.

The national tuberculosis guidelines for the programmatic management of MDR-TB in Nepal consists of an intensive phase of 8 months (with an additional 4 months if there was no culture/conversion at the end of 6 months), followed by a continuation phase of 12 months of treatment. Lfx (750 to 1,000 mg once daily) was prescribed based on body weight as described in the guidelines for management of drug-resistant tuberculosis in Nepal. Other drugs in this regimen included kanamycin (500 to 1,000 mg/day [intramuscular injection]), ethionamide (500 to 750 mg/day), pyrazinamide (20 to 30 mg/kg/day), and cycloserine (500 to 750 mg/day).

Study procedures. To assess Lfx concentrations, steady-state blood and saliva samples were collected before intake and at 1, 2, 4, and 8 h after intake of Lfx. Patients were sampled twice, i.e., at the end of the first month (days 15 to 30) and second month (days 45 to 60) of treatment. Plasma samples were collected in BD vacutainer vials (Becton Dickinson, catalog no. 23-021-016), whereas saliva samples were collected using two different techniques. Saliva samples were collected using a salivette and additionally filtered using a membrane filter (0.2- μ m diameter; Merck KGaA, Darmstadt, Germany) (29). The collected plasma/saliva samples were centrifuged and frozen at -30°C until analysis. A standardized data collection (case report forms and excel database file) was created to record demographic and clinical data of the included patients. An HIV test was carried out for all included patients as a part of the treatment protocol, but none of the included patients were HIV positive.

Drug quantification in plasma and saliva. Lfx concentrations in human plasma and saliva were analyzed in the laboratory of the Department of Clinical Pharmacy and Pharmacology at the University Medical Center, Groningen, The Netherlands, using a LC-MS/MS technique (31). The calibration curve was linear over a range of 0.10 to 5 mg/liter for Lfx. To encompass concentrations levels above 5 mg/liter, the dilution integrity was determined to accurately measure concentrations levels up to 40 mg/liter.

The pH of salivary samples was measured using pH indicator strips (Merck KGaA), encompassing a pH range from 2.0 to 9.0, with 0.5 pH unit increments distinguished by color change. Two independent observers (Samiksha Ghimire and Simone H. J. van den Elsen) recorded the results; in the case of differences, consensus was reached in the presence of a third observer (Anne-Grete Mårtson).

Data analysis. For PK parameters, noncompartmental analysis was performed using MW/Pharm (v3.82; Medware, Groningen, The Netherlands). The PK parameters included the maximal plasma concentration (C_{max}), the corresponding time of C_{max} (t_{max}), the area under plasma concentration-time curve (AUC_{0-24}), the apparent volume of distribution (V/F), the apparent clearance (CL/F), the half-life ($t_{1/2}$), and the elimination constant for plasma and saliva (k_{el}). Statistical analysis was performed using SPSS (v23.0; SPSS, Inc., Chicago, IL). The results are presented as medians with IQRs for continuous variables and number percentages for categorical variables. The normal distribution of data was ascertained by using skewness-kurtosis, visual inspection of boxplots, and the Shapiro-Wilk test. A nonparametric Wilcoxon signed rank test was used to assess the differences between plasma and saliva PK parameters, when applicable. Inter- and intraindividual pharmacokinetic variabilities were evaluated from the %CV calculated as the quotient of the standard deviation divided by the mean plasma concentration multiplied by 100. Passing-Bablok regression was used to assess the relationship between saliva and plasma Lfx concentrations (R Statistical Software). *P* values were reported as significant if *P* was <0.05 .

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