Urine Colorimetry for Levofloxacin Pharmacokinetics and Personalized Dosing in People with Drug-resistant Tuberculosis

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Abstract

Background: Levofloxacin is a preferred drug for multidrug-resistant (MDR)-tuberculosis (TB) with bactericidal activity that correlates with the pharmacokinetic exposures of serum peak concentration (C_{max}) and total area under the concentration time curve (AUC_{0-24}). Pharmacokinetic exposures can be measured to personalize dosing to reach targets, but this practice requires venepuncture, chromatographic or mass spectrometry equipment, and technical expertise. We sought to demonstrate the accuracy of using urine colorimetry as a more feasible estimation of levofloxacin exposure.

Method: A colorimetric method using bromocresol green was tested on spiked urine samples with levofloxacin measured using a spectrophotometer. This method was tested in urine samples of healthy volunteers given one 750 mg dose of levofloxacin with urine collected at 0–4 h, 4–8 h, and 8–24 h intervals, and concomitant serum samples were collected and analyzed by high-performance liquid chromatography. Validation of this assay was done in a cohort of people living with human immunodeficiency virus (PLWH), initiating a levofloxacin containing MDR-TB regimen.
Results: Urine colorimetry was reproducible in spiked samples and the calibration was curve linear for levofloxacin concentrations ranging from 7.8 μg/ml to 250 μg/ml, with $r = 0.98$. In healthy volunteers, correlation between urine absorbance values and serum AUC$_{0-24}$ was highest in urine collected between 4 and 8 h ($r = 0.91$, $P = 0.01$), yet in PLWH, urine collected between 0 and 4 h had highest correlation ($r = 0.66$, $P = 0.05$). The area under the receiver operating characteristics curve was $>0.8$ in the derivation, as well as the validation cohort for the urine absorbance values identifying people with total serum exposure below target.

Conclusion: Urine colorimetry was highly sensitive in predicting target serum concentrations. Colorimetric methods to determine levofloxacin in urine may improve the feasibility of therapeutic drug monitoring and personalized dose adjustment in TB endemic settings.

Keywords: Colorimetry; levofloxacin; multidrug-resistant tuberculosis; people living with human immunodeficiency virus; pharmacokinetics; therapeutic drug monitoring

Introduction

Tuberculosis (TB) remains the leading killer worldwide from a curable infectious pathogen. Resistance to the critical first-line drugs rifampicin and isoniazid, termed multidrug-resistant (MDR)-TB, significantly complicates treatment outcomes necessitating regimens of less efficacy and longer treatment duration.$^{[1]}$ With nearly half a million new people diagnosed with rifampicin-resistant TB in 2018, optimized treatment regimens designed and administered in TB endemic settings are urgently needed.$^{[2]}$ The World Health Organization recommendations now prioritize an all-oral medicine approach to the treatment of MDR or rifampicin-resistant TB including the class of fluoroquinolones, either levofloxacin or moxifloxacin.$^{[1]}

When ingested, the fluoroquinolones have predictable pharmacokinetics and pharmacodynamics (PK/PD) but are subject to considerable individual PK variability depending on host factors such as human immunodeficiency virus (HIV) co-infection which may predispose to malabsorption and kidney function given the fluoroquinolone’s urinary metabolism and excretion.$^{[3,4]}$ Levofloxacin, a preferred fluoroquinolone for TB treatment, exerts potent in vitro and in vivo activity against Mycobacterium tuberculosis in a concentration-dependent manner, with higher maximum concentrations (C$_{max}$) and total area under the concentration-time curve (AUC) relative to the minimum inhibitory concentration (MIC) leading to greater bacterial kill and prevention of resistance.$^{[5]}$ Among people treated for MDR-TB, it has been demonstrated that serum levofloxacin AUC/MIC ratios below certain population targets associate with treatment failure and were in fact the most important predictor of treatment outcome in adjusted analyses.$^{[6,7]}

Measurement of serum drug concentrations and adjusting the levofloxacin dose to reach a serum C$_{max}$ or AUC target is performed in certain specialized centers but requires cold transport of sera for analysis by chromatography or mass spectrometry, and those resources are often absent in TB endemic areas where such precision dosing may be most needed. In a previous study among people living with HIV (PLWH) and undergoing drug-resistant TB...
treatment in the heavily coburdened region of Irkutsk, Siberia, it was found that 11 of 30 people (37%) failed even to achieve a serum levofloxacin C<sub>max</sub> greater than the MIC.<sup>[8]</sup>

Increasingly, alternative methodologies have been utilized to overcome the barriers to personalized dosing, such as the use of urine as the analytic specimen to bypass the need for serum collection and cold transport and spectrophotometric testing (colorimetry) as a readout to eliminate the need for mass spectrometry or chromatography.<sup>[9,10]</sup> Application of urine colorimetry for assessment of rifampicin PK/PD targets has been successful.<sup>[11]</sup> Thus, we modified a previously described assay for fluoroquinolone concentration measurement by urine colorimetry, examined its performance over a 24 h dosing interval of levofloxacin excretion, and assessed its ability to predict clinically relevant serum PK/PD targets from PLWH and taking levofloxacin for MDR-TB.

**Method**

**Urine colorimetric assay**

Estimation of levofloxacin in pharmaceutical preparations has been previously demonstrated using spectrophotometry.<sup>[12,13]</sup> Similar principles were adopted to determine levofloxacin in urine by acid-dye complexation reactions using bromocresol green. To 300 μl of the urine sample, 100 μl of 0.4 M sodium acetate buffer (pH 3.8) was added and incubated for a minute, followed by the addition of 100 μl bromocresol green (1 mM dissolved in methanol). To this mixture, 500 μl of chloroform was added, and the vials were centrifuged at 13,000 rpm for 5 min. To a 96-well plate, 100 μl of the aqueous phase (upper layer) was transferred and absorbance was measured at 440 nm in a spectrophotometer (Bio-Rad iMark). A calibration curve was constructed using six two-fold serial dilutions with 250 μg/ml as the highest levofloxacin concentration.

To test for environmental effects, samples from healthy donors spiked with known concentrations of levofloxacin were stored in ambient indoor lighting/temperature, on a windowsill with direct sunlight, in a dark box at room temperature, and under refrigeration at 4°C. Chemicals were purchased from Sigma-Aldrich or Fisher Scientific, and all measurements were done in triplicates.

**Derivation study**

To study urinary kinetics of levofloxacin, a nonrandomized open-label study recruited healthy volunteers, both males and females, between the ages of 18 and 50. Eligible participants signed informed consent for the protocol approved by the institutional review board. All healthy subjects were given a single 750 mg dose of levofloxacin in a fasted state. A pharmacokinetic sampling of serum was done by collecting blood at 1, 2, and 6 h postdose. Levofloxacin concentrations in serum samples were quantified by a previously validated assay using high-performance liquid chromatography (HPLC).<sup>[14]</sup> Urine was collected at the intervals 0–4 h, 4–8 h, and 8–24 h in part to determine if there were comparably optimal collection intervals for prediction of serum PK/PD targets, and levofloxacin concentrations in urine were measured using colorimetry.
Validation study

Patients recruited from a regional referral TB hospital were 18 years or older, living with HIV and on antiretrovirals, and were 2 weeks or more from starting a levofloxacin containing regimen for MDR-TB. Patients signed written informed consent in a protocol approved by various institutional review boards. Levofloxacin dose was determined by the treating physicians and given as a directly observed oral daily administration along with other medications in the anti-TB and antiretroviral regimen. Serum collection was performed at 1, 2, 6, and an additional 8 h after the dose. Serum levofloxacin was quantified by HPLC utilizing a previously validated method.\cite{12} Urine was collected at the 0–4 h, 4–8 h, and 8–24 h intervals, similar to the derivation study, and colorimetry was performed using the above procedure. Both serum and urine levofloxacin quantification were performed onsite.

Statistical analysis

Noncompartmental analysis was performed to estimate the area under the concentration-time curve over 24 h (AUC\(_{0-24}\)) using serum concentrations in Phoenix WinNonlin version 8.0 (Certara USA, Princeton, New Jersey). Based on previous studies of PK/PD and treatment outcomes,\cite{6,7} serum levofloxacin C\(_{max}\) of 8 μg/mL and AUC\(_{0-24}\) of 80 μg × h/mL were used as the standard-of-care targets during anti-TB treatment.\cite{15} as concentrations below these breakpoints have previously predicted poor long-term treatment outcome. To determine the best time interval for the collection of urine samples, serum AUC\(_{0-24}\) was compared with urine concentrations at 0–4 h, 4–8 h, and 8–24 h using linear correlation. Further analysis was performed for urine concentrations at the time interval that exhibited the highest correlation with serum. Receiver operating characteristics (ROC) curves were generated; an optimal assay cutoff value was determined using Youden’s J index, which was subsequently used to determine the sensitivity and specificity of the colorimetric assay. Area under the ROC curve and 95% confidence interval (CI) after 2000 bootstrap replicates were determined in R software (version 3.6.1, \url{http://r-project.org}) using the pROC package.\cite{16}

Other statistical analyses were performed in Stata 15 (StataCorp, College Station, TX, USA).

Results

Colorimetric assay

A calibration curve was constructed [Figure 1] which was linear for levofloxacin concentrations ranging from 7.8 μg/ml to 250 μg/ml, with \(r = 0.98\). The performance of the assay was variable for samples exposed to different environmental conditions, except refrigeration. The mean variation over 24 h was highest when exposed to sunlight (23%), followed by ambient light (6%) and in a dark box under ambient conditions (6%). Variation was least (1%) when the vials were refrigerated at 4°C.

Derivation study

Six healthy volunteers were enrolled for levofloxacin urine kinetics [Table 1]. The median serum C\(_{max}\) was 8.30 μg/ml and the time to the maximum concentration attainment (T\(_{max}\)) was equally split between 1 and 2 h after dose administration. Three (50%) of the six...
participants attained a serum $C_{\text{max}}$ higher than the target of 8 $\mu$g/ml, and four (66.7%) achieved an $AUC_{0-24}$ higher than 80 $\mu$g $\times$ h/ml. Serum concentration characteristics were plotted against time [Figure 2]. Linear correlation between serum $AUC_{0-24}$ and urine concentrations was highest for the 4–8 h collection interval ($r = 0.91, P = 0.01$), [Figure 3] relative to 0–4 h ($r = 0.3, P = 0.57$) and 8–24 h ($r = 0.38, P = 0.53$) collection intervals. ROC curve to identify participants below an $AUC_{0-24}$ of 80 $\mu$g $\times$ h/ml resulted in an excellent area under the ROC curve of 0.88 (95% CI 0.5–1.00), [Figure 4]. For a urine concentration threshold of 197 $\mu$g/ml, the colorimetric assay was 100% sensitive and 75% specific, correctly identifying both participants with serum $AUC_{0-24}$ below target and three of four participants within the target.

**Levofloxacin quantification in people living with human immunodeficiency virus**

Data from 10 patients [Table 1] were included for the final analysis. Serum kinetics over an 8-h period was plotted [Figure 5]. Median serum $C_{\text{max}}$ and $AUC_{0-24}$ were 8.30 $\mu$g/ml and 77.40 $\mu$g $\times$ h/ml, respectively. $T_{\text{max}}$ after dose administration was 1-h in five participants (50%), 2-h in three (30%), and at 6-h in two (20%). A serum $C_{\text{max}}$ target >8 $\mu$g/ml and serum $AUC_{0-24}$ target >80 $\mu$g $\times$ h/ml were attained in six (60%) and five (50%) patients, respectively. A linear correlation was performed between serum $AUC_{0-24}$ and urine concentrations at different collection intervals. Contrary to the results in the derivation cohort, correlation was highest at the 0–4 h urine collection interval ($r = 0.66, P = 0.05$), [Figure 6] compared to the 4–8 h ($r = 0.42, P = 0.23$) and 8–24 h ($r = 0.26, P = 0.5$) collection intervals. ROC analysis with urine concentrations at 0–4 h collection interval and serum $AUC_{0-24}$ resulted in an area under the ROC curve of 0.85 (95% CI: 0.5–1.00), [Figure 7]. At a threshold of 261.4 $\mu$g/ml, the assay was 100% sensitive and 50% specific, correctly identifying six participants below and two participants above target serum $AUC_{0-24}$ 80 $\mu$g $\times$ h/ml.

**Discussion**

We were successfully able to quantify levofloxacin in urine with a spectrophotometer using colorimetric principles. Although urine has been used for testing the presence of some first-line TB drugs using colorimetry,[11,14] to the best of our knowledge, this is the first time levofloxacin has been quantified in urine by colorimetric principles obtained from healthy participants after levofloxacin administration and patients living with HIV treated with levofloxacin for MDR-TB. The area under the ROC curve was >0.8 in the derivation as well as the validation study, which demonstrates that this assay performs well in predicting clinically important serum targets.[17] The urine colorimetric assay was particularly sensitive in predicting serum values that were below target and which would necessitate dose increase.

Of further clinical relevance, favorable urine collection intervals for testing levofloxacin absorbance could either be from 0 to 4 h or 4–8 h. While these findings were not consistent between the population of PLWH and treated for MDR-TB and the healthy controls, it is likely that PLWH had more variable gastrointestinal absorption and were taking concurrent antiretroviral therapy and other anti-TB medications that affected not only the total drug
exposure, AUC\textsubscript{0-24} but also the timing or urine accumulation of levofloxacin. Our findings highlight the importance of performing studies among populations with TB and relevant comorbidities. Future studies should focus on collecting urine samples at specific spot time points to determine if feasibility can be further improved whereby urine could reasonably be collected in a single clinic visit.

Although the majority of PLWH and treated for MDR-TB had adequate levofloxacin dose per body weight, target $C_{\text{max}}$ was attained only in six patients (60%). Previous studies have demonstrated low peak serum levofloxacin values in patients with MDR-TB and HIV/AIDS as compared to healthy controls.\textsuperscript{[18,19]} Yet even among healthy volunteers in this study, we observed significant interindividual pharmacokinetic variability. While we did not measure MIC values for levofloxacin from sputum cultures of \textit{M. tuberculosis} among the PLWH and treated for MDR-TB, MIC values may vary within the infecting \textit{M. tuberculosis} population and the anatomic location within the host. Fortunately, for the fluoroquinolones, human studies demonstrate a dose correlation with serum concentrations, and dose-dependent increases in concentrations within \textit{M. tuberculosis}-infected lung lesions, suggesting that increasing serum concentrations to improve AUC/MIC or $C_{\text{max}}$/MIC relationships will lead to better activity at the site of pulmonary infection.\textsuperscript{[20,21]} Thus, even in the absence of MIC values, our findings suggest dose adjustments of levofloxacin based on urine colorimetry could significantly optimize PK/PD relationships in settings without access to serum HPLC or mass spectrometry.

The colorimetric assay did not have a wide quantification limit for all typically observed urine concentrations for levofloxacin within a dosing interval, with 250 μg/ml being the highest and 7.81 μg/ml as the lowest limit of quantification. Yet, this limitation was overcome with the dilution of samples in a 1:1 ratio. Importantly, the analysis performed on spiked urine samples resulted in variability in unrefrigerated samples exposed to various environmental conditions which in prolonged sunlight, for instance, may be unacceptable for implementation. Unless samples are analyzed in real-time after collection, our studies suggest that refrigeration after collection may lessen variability in results, but further testing is needed before determining the acceptable range of transportation and storage conditions.

Personalized dose adjustment based on an individual’s PK aligns with scientific evidence that pharmacokinetic variability is principally responsible for TB treatment failure and acquired resistance and less so patterns of adherence or programmatic deficits.\textsuperscript{[22]} As such, urine colorimetry may offer a person-centered approach to regimen construction that could improve individual outcomes and also offer another important means of pharmacovigilance to ensure the effectiveness of the background regimen upon which new drugs for MDR-TB, such as bedaquiline, are added.\textsuperscript{[23]}

**Conclusion**

Colorimetric principles were successfully implemented in quantifying levofloxacin concentrations in urine. The urine colorimetric assay was highly sensitive in predicting low serum concentrations. Given pharmacokinetic variability being is a major contributor to
unfavourable outcomes among people with MDR-TB, the simple spectrophotometric procedures may enhance personalised dosing in TB endemic settings.

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Figure 1:
Calibration curve for levofloxacin colorimetric assay
Figure 2:
Serum pharmacokinetics in healthy volunteers
Figure 3:
Correlation between serum AUC$_{0-24}$ (µg × h/ml) and urine levofloxacin concentration (µg/ml) at 4–8 h interval
Figure 4:
Receiver operating characteristics curve to identify participants with low serum levofloxacin AUC$_{0-24}$ (μg × h/ml) in the derivation study.
**Figure 5:**
Serum pharmacokinetics of tuberculosis patients in the validation cohort
Figure 6:
Correlation between serum AUC0-24 (µg × h/ml) and urine levofloxacin concentration (µg/ml) at 0–4 h interval
Figure 7:
Receiver operating characteristics curve to identify patients with low serum levofloxacin AUC_{0-24} (μg × h/ml) in the validation study.
**Table 1:**
Baseline characteristics of participants from derivation and validation study

| Characteristic                        | Healthy participants (n=6) | People with MDR-TB (n=10) |
|---------------------------------------|---------------------------|---------------------------|
| Female                                | 4 (66.7%)                 | 3 (33.3%)                 |
| Age                                   | 29 (25.3-33.5)            | 36.5 (29.5-41)            |
| Weight (kg)                           | 81.6 (77.6-83.9)          | 56.5 (49.3-61.5)          |
| Dose (mg/kg)                          | 9.2 (8.9-9.7)             | 12.3 (10.9-13.7)          |
| Maximum serum concentration (µg/ml)   | 8.3 (7.6-9.3)             | 8.3 (5.4-9.7)             |
| Serum AUC_{0-24} (µg*h/ml)            | 91.7 (73.9-102.5)         | 77.4 (54.6-105.6)         |

IQR: Interquartile range, AUC_{0-24}: Total area under the concentration time curve