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Cross validation of a liquid chromatography tandem mass spectrometry method for quantification of levofloxacin in saliva

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ABSTRACT

Cross validation studies ensure multi-purpose use of bioanalytical methods for the measurement of drug concentrations across different matrices. In this study, we cross validated a previously developed liquid-chromatography tandem mass spectrometry method for the quantification of levofloxacin in serum for saliva as matrix using serum standards. The standard curve was linear within the concentration range of 0.20–50 mg/L for levofloxacin in both serum and saliva. Bias for saliva quality control samples were −1.0 %, −0.9 %, −0.3 % and 2.0 % at lower limit of quantification (LLOQ), low, medium and high concentrations, whereas coefficient of variation (CV) were 2.3 %, 1.0 %, 0.9 %, and 1.8 % at LLOQ, low, medium and high concentrations respectively. Therefore, concentrations of levofloxacin in human saliva can be quantified using calibration standards in human serum. The mean recovery of levofloxacin in saliva using plain cotton rolls along with CV was around 70 % and 7 % at both 1 and 5 mg/L.

INTRODUCTION

Saliva could be a potential alternative sampling matrix for measurement of drug concentrations in patients for routine clinical care (1,2). In recent years, liquid chromatography tandem mass spectrometry methods (LC-MS/MS) have been developed for detection of levofloxacin along with other anti-tuberculosis (anti-TB) drugs in human plasma/serum (3–6). However, measurement of these drugs in saliva using an LC-MS/MS method developed for plasma/serum require cross validation.

The handling of infectious saliva samples from TB patients puts health care workers at risk of contagion. Therefore, membrane filtration was found to be suitable for sterilization of saliva samples, before analyzing for therapeutic drug monitoring purposes (7). However, it is likely that some of the drugs are bound to the cotton rolls used for the collection of saliva and/or membrane of the filter used during the filtration process leading to lower recoveries.

The aims of this study were: a) to assess if drug concentrations in human saliva could be determined with calibration samples prepared in human serum; and b) to perform a recovery test for levofloxacin concentrations in saliva after using sorbent material such as cotton rolls and/or filtering through a membrane filter.

MATERIALS AND METHODS

Chemicals and reagents

Levofloxacin and [$^{2}H_{4}$] levofloxacin (internal standard) were purchased from Sigma-Aldrich, MO, USA and Alsachim, IIlkrich, France. Ammonium formate buffer was made with ammonium formate obtained from Arcos Organics, NJ, USA and formic acid obtained from Merck, NJ, USA. Similarly, methanol was purchased from Merck, NJ, USA. Polyvinylidene fluoride membrane filters with a pore size of 0.22 µm and a diameter of 33 mm (Millex-GV) were purchased from Merck Millipore, Ireland. Plastic syringes (5 ml) with Luer-lock™ tips were purchased from Becton Dickinson and Company, New Jersey, USA. Dental cotton rolls no. 2 were obtained from Dynarex, NY, USA.

Preparation of stock solutions, calibration standard and quality control samples

Levofloxacin stock solution of 2.5 mg/mL was prepared in dimethyl sulfoxide (Merck, NJ, USA). Two batches of stock solutions were made. One batch was used to prepare the calibration samples in blank human serum to make nine different concentrations of 0.20, 0.50, 1, 2, 5, 10, 20, 40, and 50 mg/L for the calibration curve. The other batch was used to prepare four different concentrations of quality control samples (QC) in saliva, with a lower limit of quantification of 0.2 mg/L, low QC was 1 mg/L, medium was 20 mg/L and high was 40 mg/L. The internal standard solution was prepared from a 1 mg/ml stock solution of [$^{2}H_{4}$] levofloxacin in DMSO by diluting 50 µl to 250 ml with methanol to a concentration of 0.2 mg/L. For cross validation, all samples were analyzed in quintuplicate.

Sample preparation, processing and assay conditions

A working solution of 0.25 mg/mL was prepared by diluting the stock solution (2.5 mg/ml) ten-fold with water. A saliva standard of 0.2 mg/L was prepared by diluting 4 µL of working solution to a total volume of 5 mL with blank saliva. Standards 0.5, 1 and 2 mg/L were prepared by diluting 5, 10 and 20 µl of working solution respectively, to a total volume of 2.5 mL with blank saliva. Standard 5, 10, 20 and 40 mg/L were prepared by diluting 5, 10, 20 and 40 µL of stock solution respectively, to a total volume of 2.5 mL with blank saliva. Standard 50 mg/L was prepared by diluting 40 µL of stock solution to a total volume of 2 mL with blank saliva. In addition, quality control samples at LLOQ, Low, Medium and High were prepared identical to standards at 0.2, 1, 20 and 40 mg/mL but from a different batch of stock solution. 100 µl of standards or QC samples was aliquoted in a 2 ml vials. Then, 500 µl of internal standard was added. The mixture was vortexed for 1 minute and centrifuged at 9,500 g for 5 minutes after which 0.5 µl of supernatant was injected into the LC-MS/MS system.

The analysis was performed on a triple quadrupole LC-MS/MS (Thermo Scientific TSQ Quantiva, San Jose, CA, USA). A Thermo Accucore C18 analytical column of particle size 2.6 µm, 50 mm length and internal diameter of 2.1 mm was used. The autosampler was a Thermo Scientific Vanquish with tray temperature set at 10 °C. Mobile phase

Table 1: Cross-validation results

CRITERIA	QC concentration level			
	LLOQ	LOW	MEDIUM	HIGH
Nominal concentration	0.20 mg/L	1 mg/L	20 mg/L	40 mg/L
CV%				
Serum	3.5	6.5	3.1	3.4
Saliva	2.3	1.0	0.9	1.8
Bias %				
Serum	−4.0	−1.8	2.4	−0.7
Saliva	−1.0	−0.9	−0.3	2.0

A1 consisted of 0.02 mol/L ammonium formate buffer at pH 3.5 and mobile phase B consisted of methanol. Quantifications were achieved in the positive ion electrospray mode by Selected Reaction Monitoring (SRM). The analysis was developed and validated for use in human serum/plasma (6). The linearity of calibration curve was 0.20–50 mg/L for levofloxacin in both serum and saliva. Bias and CV for serum quality control samples at LLOQ, low, medium and high are shown in Table 1.

Cross Validation

QC samples in saliva at four concentration levels (0.20, 1, 20, 40 mg/L) were compared with levofloxacin calibration standards prepared in human plasma. Accepted bias and coefficient of variation (CV) were ≤15 % for QC samples at low, medium and high concentrations and ≤20 % for LLOQ sample in saliva (Table 1).

Recovery experiment for sample collection

Saliva samples were collected from 10 healthy volunteers. Levofloxacin stock solution of 150 mg/ml was prepared in methanol. Subsequently, two different levofloxacin concentrations of 1 mg/L and 5 mg/L were prepared by adding stock solution to saliva. The recovery of levofloxacin

in saliva was evaluated in four different test designs. The first group (blank syringe), was blank saliva which was absorbed by the cotton roll and afterwards compressed in a syringe. The effluent was then spiked with levofloxacin at 1 and 5 mg/L. In the second group (test solution syringe), levofloxacin spiked saliva at concentration of 1 mg/L and 5 mg/L were applied to the cotton rolls. The volume required to saturate the cotton rolls was determined beforehand. Thereafter, cotton rolls with absorbed saliva were compressed in a syringe by pushing the plunger of the syringe and collecting the effluent. The recovery was evaluated in the effluent. The third group (blank syringe filter) was similar to the first group, except the blank saliva was pushed through the syringe equipped with a 0.22 µm membrane filter, and later spiked with levofloxacin at the above-mentioned concentrations. In the fourth group (test solution syringe filter), recovery yield was determined after compressing fully saturated cotton rolls with levofloxacin spiked saliva at (1 mg/L and 5 mg/L) in a syringe equipped with a 0.22 µm membrane filter. The schematic representation of four test designs is shown (Figure 1).

RESULTS

The calibration curve was linear in a concentration range of 0.20–50 mg/L with a coefficient of determination (R^2) of 0.999. To compare QC samples of levofloxacin in serum with QC samples of levofloxacin in saliva, bias and CV were calculated (Table 1). Both bias and CV were ≤15 % for QC at low, medium and high and ≤20 % for LLOQ. The LC-MS/MS method had a run time of 2 min and levofloxacin eluted at a retention time of 0.7 min. The mean recovery of levofloxacin along with CV for the second (test solution syringe) test-group calculated relative to first test-group and the fourth (test solution syringe filter) test-group calculated relative to third test-group is shown in Table 2.

Clinical application of the method

This method was used for the analysis of levofloxacin concentrations in saliva samples at the laboratory of the department of Clinical Pharmacy and Pharmacology in the University Medical Center Groningen for a clinical trial (identifier number NCT 03000517) on the

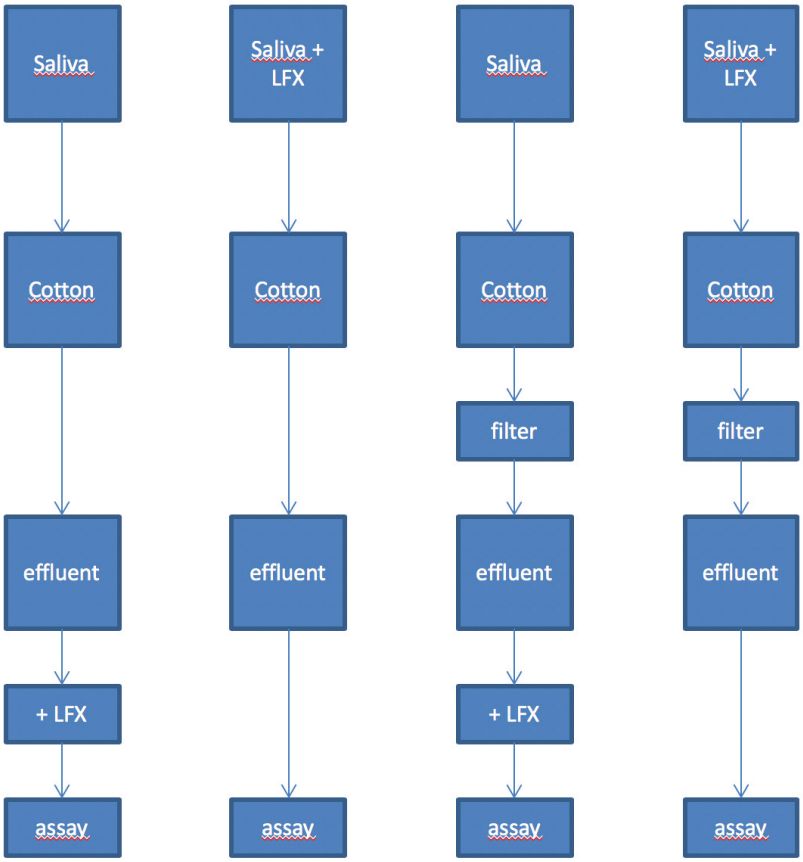

Figure 1: Schematic representation of four test designs of recovery experiment (left to right)

Table 2: Mean recovery of levofloxacin in saliva

Test group	Design	Mean recovery %	CV%
Second	Test solution syringe		
	1 mg/L	67.18	9.47
	5 mg/L	63.78	6.00
Fourth	Test solution syringe filter		
	1 mg/L	68.23	5.25
	5 mg/L	80.15	5.98

pharmacokinetics of levofloxacin in saliva of 23 MDR-TB patients. The median observed AUC_{0-24} and C_{max} were 67.09 mg*h/L and 7.03 mg/L (8).

DISCUSSION

Our findings suggest that salivary levofloxacin concentrations could be determined using calibration standards in human serum using an LC-MS/MS method developed and validated for plasma/serum (6). The method is cross-validated according to the Bioanalytical method validation: Guidance for industry, as specified by FDA (9). This method can inspire bioanalytical laboratories across the globe to cross validate an LC-MS/MS method developed in plasma/serum for several other drugs in saliva.

Furthermore, this study has shown that the plain cotton rolls achieved a recovery of around 70 %. This will have an impact on the variability of analytical results with an spread of 17 % and bias of approximately 30 %, if cotton rolls are used as a sampling device. This is likely due to sorption of levofloxacin to the cotton roll. Therefore, saliva samples could be useful only in screening and semi-quantitative prediction of plasma levels of TB drugs. In addition, our experiments have shown that filtration through a 0.22 µm filter does not result in a further loss of levofloxacin. Such recovery experiments should be performed for other drugs of interest, if cotton rolls are used in clinical practice to collect saliva samples.

CONCLUSION

Results of the cross-validation study were within the acceptance criteria for bias and precision according to formal regulations. Therefore, the LC-MS/MS method initially developed for quantifying levofloxacin concentrations in serum could be used to determine its concentrations in saliva. The cotton rolls used for saliva sample collection achieved a levofloxacin recovery of around 70 %.

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