

A SIMPLE AND RAPID LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF LEVOFLOXACIN IN PLASMA

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ABSTRACT

Introduction: Levofloxacin (LFX) is one of the second line anti-tuberculosis drugs used in the treatment of multi drug resistant tuberculosis. Monitoring of LFX concentrations in plasma may be valuable to study its pharmacokinetics and drug-drug interaction when co-administered with other anti-tuberculosis drugs. We developed a high performance liquid chromatographic method of determination of LFX in plasma.

Methodology: The method involved deproteinisation of the sample with perchloric acid and analysis of the supernatant using a reversed-phase C₁₈ column (150mm) and fluorescence detection at an excitation wavelength of 290 nm and an emission wavelength of 460 nm.

Results: The assay was specific for LFX and linear from 0.25 to 10.0 µg/ml. The relative standard deviation of intra- and inter-day assays was lower than 10%. The average recovery of LFX from plasma was 99%.

Conclusion: A sensitive, specific and validated method for quantitative determination of LFX in plasma was developed. Due to its simplicity; the assay can be used for pharmacokinetic studies of LFX.

Key words: Levofloxacin, Plasma, HPLC, Fluoroquinolones

INTRODUCTION

Tuberculosis remains one of the main causes of mortality and morbidity worldwide. The emergence of multi-drug resistant strains of *M. tuberculosis* strains in some parts of the world has become a major concern. The anti-tuberculosis activity of the fluoroquinolones has been under investigation since the 1980s.¹ Levofloxacin (LFX) represents one of the few second-line drugs introduced in the therapeutic regimens for Multi-drug resistant tuberculosis.² Studies have shown that LFX has higher *in vitro* activity compared to older fluoroquinolones and was well-tolerated and safe in multi-drug resistant tuberculosis patients.²

Both levofloxacin and moxifloxacin were shown to possess equivalent efficacy for treating multi-drug resistant tuberculosis.³ Monitoring of LFX concentrations in plasma may be valuable to study its pharmacokinetics and drug-drug interactions when co-administered with other anti-tuberculosis drugs.

Several high performance liquid chromatography methods using both fluorescence and ultraviolet detectors have been developed for measuring plasma LFX concentrations.⁴⁻¹⁶ While few methods are simple, some of the methods are quite cumbersome and time consuming. These methods have not checked for interference of anti-tuberculosis drugs in their specificity experiment. Since LFX is used along with anti-tuberculosis drugs, it is essential to rule out interference of these drugs in the assay of levofloxacin. We developed and validated a simple and rapid assay procedure for estimation of LFX in plasma based on the method that we had earlier developed for ofloxacin and moxifloxacin.^{17,18}

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METHODOLOGY

Pure LFX powder was purchased from Sigma Aldrich Chemical Company, MO, USA, moxifloxacin from Selleck Chemicals LLC, USA, acetonitrile (HPLC grade) from Merck (India), potassium dihydrogen orthophosphate and perchloric acid from Qualigens (India) were used. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human plasma was obtained from Lions Blood Bank, Chennai, India.

Chromatographic System

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), fluorescence detector (RF-10AXL) and auto sampler (SIL-HTA) with built in system controller. Class VP-LC workstation was used for data collection and acquisition. The analytical column was a C18, 150 mm ×4.6 mm ID, 5 µm particle size (Lichrospher 100 RP-18e, Merck, Germany) protected by a compatible guard column.

The mobile phase consisted of 0.05 M phosphate buffer, pH 2.6 (adjusted with 1 N hydrochloric acid) and acetonitrile (80:20, v/v). Prior to preparation of the mobile phase, the phosphate buffer and acetonitrile were degassed separately using a Millipore vacuum pump. The fluorescence detector was set at an excitation wavelength of 290 nm and an emission wavelength of 460 nm. The chromatogram was run for 7 minutes at a flow rate of 1.2 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis of the peak height ratios (analyte/internal standard) vs. concentration curve. The linearity was verified using estimates of correlation coefficient (*r*).

Preparation of standard solution

A stock standard (1 mg/ml) was prepared by dissolving LFX in 0.1N hydrochloric acid. The working standards of LFX in concentrations ranging from 0.25 to 10.0 µg/ml were prepared in pooled plasma.

Sample preparation

To 50 µl each of calibration standards and test samples (from healthy volunteers), 10 µl of moxifloxacin (internal standard) was added at a

concentration of 100µg/ml. This was mixed with 25µl of 7% perchloric acid, the contents were vortexed vigorously, and centrifuged at 10,000 rpm for 10 min. 20 µl of the clear supernatant was directly injected to the HPLC column.

Accuracy and Linearity

The accuracy and linearity of LFX standards were evaluated by analysing a set of standards ranging from 0.25 to 10.0µg/ml. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

Precision

In order to evaluate the precision of the method, three different plasma samples from healthy subjects containing varying concentrations of LFX were analysed in duplicate on three consecutive days.

Recovery

For the recovery experiment, known concentrations of LFX (0.25, 0.5, 2.5, 5.0 & 10.0 µg/ml) were prepared in pooled human plasma samples and were spiked with 0.5, 1.25 and 2.5 µg/ml LFX and assayed after addition of the internal standard. The percentage of recovery was calculated by dividing sample differences with the added concentrations. Recovery experiments were carried out on three different occasions.

Interference from endogenous compounds was investigated by analysing blank plasma samples obtained from six each of male and female subjects. Interference from certain anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, ethionamide, cycloserine and certain antiretroviral drugs, namely, nevirapine, efavirenz, zidovudine, didanosine, stavudine, lamivudine, saquinavir, lopinavir, ritonavir and indinavir at a concentration of 10µg/ml was also evaluated.

Limits of quantification (LOQ) and detection (LOD)

These values were estimated mathematically from the standard curve equations. The LOQ was obtained by multiplying the standard deviation (SD) of the Y-axis intercepts by 10. The LOD was equal to 3.3 times the SD of the Y-axis intercepts 19.

Samples

The method developed and validated was applied in plasma samples obtained from multi drug resistant tuberculosis patients who receive LFX as part of their anti-tuberculosis treatment. Blood samples were collected from these patients, who were admitted in the Government Hospital of Thoracic Medicine, Tambaram, Chennai for the pharmacokinetic study. Their age and body weight ranged from 35 to 60 years and 48 to 70kg respectively. These patients are administered with 1000mg LFX along with other second line anti-tuberculosis drugs. Two milliliters of blood was collected at two hours after directly observed drug administration in a heparinised vacutainer tube. Plasma was separated and stored at -20°C. Estimation of plasma LFX was undertaken within 48 hours of blood collection. The study commenced after obtaining approval from the Institutional Ethics Committee. Informed, written consent was obtained from the study patients before they took part in the study.

RESULTS AND DISCUSSION

In this study, sample preparation required a simple one-step deproteinisation method and analysis using a C₁₈ column and an isocratic mobile phase. The present method has the advantages of being rapid (run time is only 7 minutes) and using a small sample volume (50 microlitres), without any loss of analyte. The use of internal standard helped in monitoring the recovery of LFX from plasma. Moxifloxacin was chosen as the internal standard since the present method was a modification of an earlier method that we had developed for estimation of moxifloxacin in plasma and urine¹⁸, and it had a different retention time to that of LFX.

Under the chromatographic conditions described above, LFX was well separated as seen in the representative chromatograms (Figure 1a, b). The retention times of LFX and internal standard were 1.9 and 4.5 minutes respectively. Blank plasma samples did not give any peak at the retention times of LFX and moxifloxacin (Figure 1c). The lowest concentration of LFX gave a discrete peak at 4.5 minutes (Figure 1a). A representative chromatogram of a healthy volunteer's plasma sample following extraction and analysis is shown in Figure 1d.

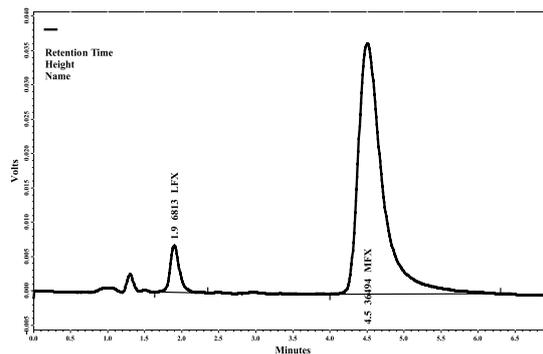


Figure 1a. Chromatogram of extracted LFX plasma standard 0.25 µg/ml (contains internal standard - 10 µl of moxifloxacin added at a concentration of 100µg/ml)

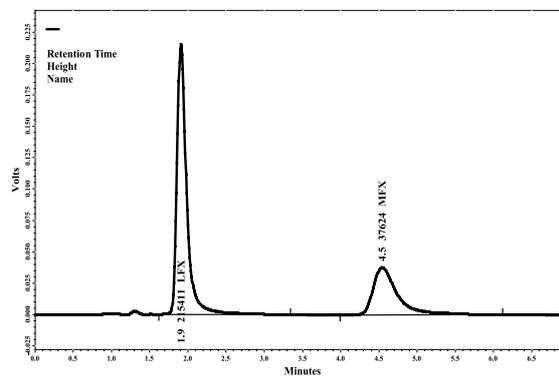


Figure 1b. Chromatogram of extracted LFX plasma standard 10.0 µg/ml (contains internal standard - 10 µl of moxifloxacin added at a concentration of 100µg/ml)

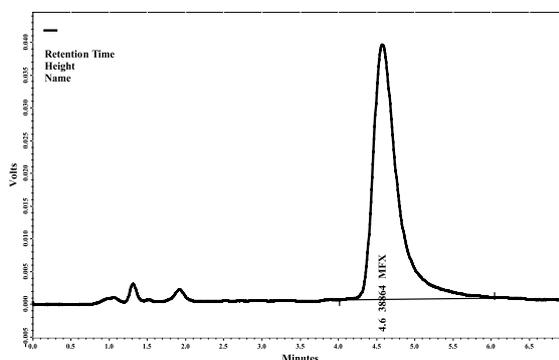


Figure 1c. Chromatogram of extracted blank plasma (contains internal standard - 10 µl of moxifloxacin added at a concentration of 100µg/ml)

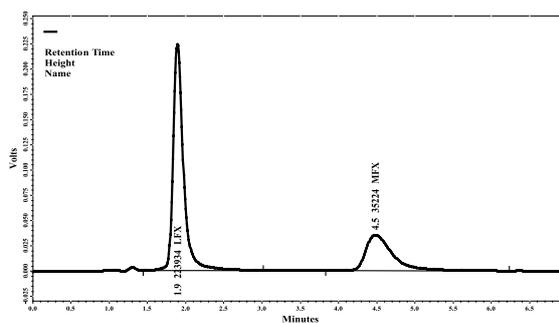


Figure 1d. Chromatogram of extracted healthy volunteer plasma (contains internal standard - 10 µl of moxifloxacin added at a concentration of 100µg/ml)

In view of its potent anti-mycobacterial activity, LFX is used in the treatment of tuberculosis along with first and second line anti-tuberculosis drugs. It, therefore, becomes necessary to rule out interference of anti-tuberculosis drugs in the assay of LFX and establish the specificity of the method. No endogenous substances or anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, ethionamide, cycloserine or antiretroviral drugs such as nevirapine, efavirenz, zidovudine, didanosine, stavudine, lamivudine, saquinavir, lopinavir, ritonavir and indinavir interfered with the LFX chromatogram.

The minimal inhibitory concentration (MIC_{90}) of LFX against *M.tuberculosis* is 0.5 μ g/ml.²⁰ After a daily oral dose of 1000 mg LFX, the mean maximum plasma concentration of LFX at steady state is 8.24 μ g/ml; this is attained at 2 hours post-dosing.²¹ In the present method, LFX concentrations ranging from 0.25-10.0 μ g/ml were checked for linearity. These concentrations span the range of clinical interest, the lowest concentration of 0.25 μ g/ml being lower than the MIC of the drug. The calibration curve parameters of levofloxacin from six individual experiments for standard concentrations ranging from 0.25 to 10.0 μ g/ml showed a linear relationship between peak height ratio and concentrations (Figure 2).

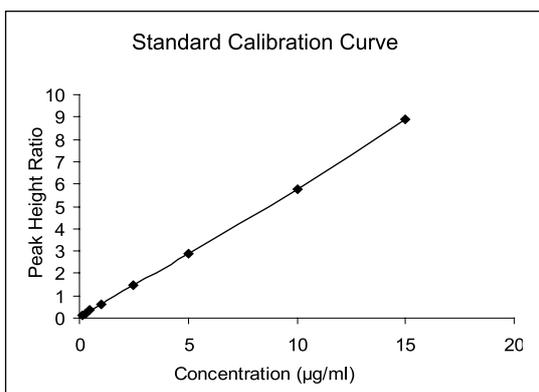


Figure 2. The calibration curve parameters of LFX from six individual experiments for standard concentrations ranging from 0.25 to 10.0 μ g/ml showed a linear relationship between peak height ratio and concentrations.

The mean (\pm SD) correlation coefficient, slope and intercept values were 0.9990 ± 0.0008 , 0.6890 ± 0.2504 and 0.0260 ± 0.0115 respectively. The linearity and reproducibility of the various standards used for constructing calibration graphs for plasma LFX are given in Table 1.

Table 1. Linearity and reproducibility of plasma LFX standards

Standard Concentration (μ g/ml)	Mean peak height ratio \pm SD (RSD %)	
	Within day (n=6)	Between day (n=6)
0.25	0.17 \pm 0.01 (5.3)	0.18 \pm 0.01 (5.6)
0.5	0.31 \pm 0.01 (3.2)	0.34 \pm 0.03 (7.8)
1.0	0.62 \pm 0.01 (2.0)	0.65 \pm 0.03 (5.0)
2.5	1.59 \pm 0.03 (1.6)	1.63 \pm 0.11(6.6)
5.0	3.00 \pm 0.06 (1.9)	3.11 \pm 0.19(6.1)
10.0	6.08 \pm 0.09 (1.5)	6.21 \pm 0.34(5.4)

The within-day and between-day relative standard deviation (RSD) for standards containing 0.25 to 10.0 μ g/ml ranged from 2.5 to 7.8% and 5.0 to 7.8% respectively.

The reproducibility of the method was further evaluated by analysing three plasma samples from healthy subjects containing different concentrations of LFX. The RSD for these samples ranged from 1.9 to 6.1% (Table 2).

Table 2: Precision of plasma LFX assay

	Sample 1	Sample 2	Sample 3
	7.48	5.21	3.21
	7.55	5.55	3.25
	7.15	5.89	3.33
Mean	7.39	5.55	3.26
SD	0.21	0.34	0.06
%RSD	2.89	6.13	1.87

The % variations from the actual ranged from 96 to 106%. The LOD and LOQ estimated mathematically from the standard curve equation¹⁹ were 0.04 μ g/ml and 0.12 μ g/ml respectively. The method reliably eliminated interfering material from plasma, yielding a recovery for LFX that ranged from 96 to 104%.

The method described was applied for the determination of LFX concentration in plasma from 10 healthy subjects who received a single oral dose of 500mg LFX. A mean plasma peak concentration of 5.61 μ g/ml was obtained at two hours, the range being 3.21 to 8.03 μ g/ml. This value is similar to that reported by Tsaganos and others.²¹ The assay spans the concentration range of clinical interest.

Several HPLC methods have been described to measure LFX levels in plasma for pharmacokinetic studies. The sample preparation used in the method described by Zhou *et al.*⁴ involves liquid-liquid extraction, evaporating the organic phase to dryness and reconstituting the dried residue in

the mobile phase. This could be time consuming compared to the one-step sample preparation procedure that we describe here. Siewert has used a gradient mobile phase, which could be quite complex compared to an isocratic mobile phase as described in our method.⁷ Methods using liquid-solid extraction using oasis cartridges have also been reported¹⁵; this could be quite expensive. Other methods have used pre-column processing⁸ or ultrafiltration⁹ for estimation of plasma LFX.

CONCLUSION

A sensitive, specific and validated method for quantitative determination of LFX in plasma is described. This rapid, accurate and reproducible method utilises a single step extraction. The chromatogram yields a well-resolved peak for LFX with good intra- and inter-day precision. The easy sample preparation and small sample size makes this assay highly suitable for pharmacokinetic studies of LFX in tuberculosis patients.

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