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*Full Length Research Paper*

# Development and validation of RP-HPLC assay for levofloxacin in rat plasma and saliva: Application to pharmacokinetic studies

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**A new, simple, specific, accurate and precise reversed phase-high performance liquid chromatography (RP-HPLC) method was developed for the determination of levofloxacin in rat plasma and saliva was developed. An HPLC system based on a Phenomenex Luna $C_{18}$ Column (250 × 4.6 mm) and a UV detector (λ = 296 nm) were used. A mixture of Acetonitrile: water (80:20 *v/v*) adjusted to pH 3.5 by orthophosphoric acid at a flow rate of 1.4 ml/min was used as mobile phase. The proteins were precipitated with methanol. The average recovery was 94.79 and 92.66%, respectively in plasma and saliva. The detection limit for levofloxacin in plasma and saliva was 1 µg/ml. The calibration curve was linear over the concentration range 1 to 16 µg/ml for plasma and saliva. The inter-day and intra-day assay coefficients of variation were found to be less than 5%. The present validated method was successfully used for pharmacokinetic studies of levofloxacin in plasma and saliva.**

**Key words:** High-performance liquid chromatography (HPLC), levofloxacin, plasma, saliva, pharmacokinetics.

## INTRODUCTION

Levofloxacin (LOFLX), (–)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid, is an advanced-generation fluoroquinolone antibiotic. It has broad-spectrum *in vitro* activity, including activity against many clinically encountered Gram positive and Gram negative organisms (Fu et al., 1992), and is therefore administered to treat various infectious diseases, like community acquired and nosocomial pneumonia, skin and skin structure infection, urinary tract infections or sepsis (North et al., 1998). Among different types of chromatographic methods, high performance liquid chromatography (HPLC) is found to be more effective to achieve separation, identification, purification and quantification of various compounds. Applicability of HPLC in pharmaceutical and biomedical analysis has increased during the last three decades and also for simultaneous determination in biological fluids (Muhammad et al., 2011). Although, several HPLC methods coupled with different detection techniques have been reported for quantification of levofloxacin in blood plasma (Siewert, 2006; Djabarouti et al., 2004). Drug monitoring using the saliva offers a convenient and non-invasive 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). 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 this dose (Albarellos et al., 2005). Therefore, the objective

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**Abbreviations: LOFLX,** Levofloxacin; **HPLC,** high-performance liquid chromatography.

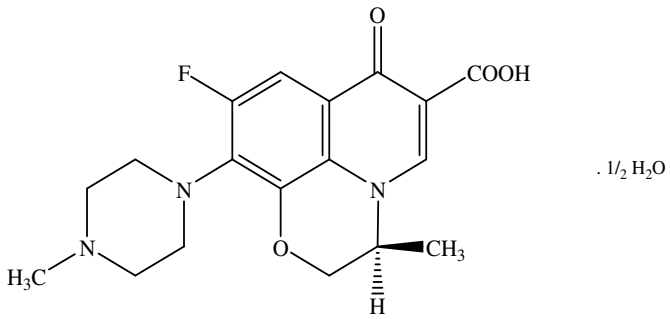


**Figure 1.** Structure of levofloxacin.

of study was to develop a new, simple, specific, accurate and precise RP-HPLC method for quantification of LOFLX (Figure 1) in plasma and saliva.

## EXPERIMENTAL

### Instrumentation

The HPLC method was performed on a Shimadzu Class LC-10AT fp and LC-20AD pumps connected with SPD-10A vp UV-Visible detector. The data acquisition was performed by Spincotech software version 1.7. The method was conducted using a reversed-phase technique. The UV Winlamb version 2.8.04 software was used for all absorbance measurements.

### Chemicals

Levofloxacin hemihydrate (analytical grade) was kindly supplied by Micro Labs. Ltd., Pondicherry, India. Acetonitrile (HPLC) and methanol was purchased from Rankem chemical, Delhi, India. Orthophosphoric acid was purchased from Ranbaxy fine chemicals limited, New Delhi, India. Heparin was obtained from Sisco Research Lab Pvt. Ltd, Mumbai, India.

### Chromatographic condition

The following optimized chromatographic parameters were used for the estimation of LOFLX in plasma and saliva:

- Mode of operation: Isocratic
- Stationary phase: Phenomenex Luna $C_{18}$ Column (250 × 4.6 mm) 5 µ particle size
- Mobile phase: Acetonitrile: Water
- Mobile phase pH: Orthophosphoric acid (pH 3.5)
- Mobile phase Ratio: 80: 20
- Detection wavelength: 296 nm
- Flow rate: 1.4 ml/min
- Temperature: Ambient
- Pressure: 100 kgf
- Injection volume: 20 µl

### Sample preparation

#### *Sample extraction*

To 0.2 ml of heparinized plasma, 0.2 ml of 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.

#### *Plasma calibration and quality control*

The stock solution of LOFLX was prepared by dissolving the appropriate amount of LOFLX, accurately weighed in methanol to yield a final drug concentration of 100 µg/ml. Working stock solutions of 16, 8, 4, 2 and 1 µg/m were prepared by appropriate dilution with mobile phase. Then, each working stock solution was diluted into free plasma to obtain a concentration range from 1 to 16 µg/ml. Aliquots of those solutions were stored at -20°C.

#### *Saliva calibration and quality control*

The stock solution of LOFLX was prepared by dissolving the appropriate amount of LOFLX, accurately weighed in methanol to yield a final drug concentration of 100 µg/ml. Working stock solutions of 16, 8, 4, 2 and 1 µg/ml were prepared by appropriate mobile phase. Then, each working stock solution was diluted into free rat saliva to obtain a concentration range from 1 to 16 µg/ ml. Aliquots of those solutions were stored at -20°C.

### Validation

The method was validated in accordance with Food and Drug Administration (FDA) guidelines (Siewert, 2006).

#### *Calibration plot*

The calibration plot for the HPLC method was constructed by analysis of five solutions containing different concentrations of levofloxacin (16, 8, 4, 2 and 1 µg/ ml) in plasma and saliva. The data were best fitted by a linear equation $mx + b = y$, the correlation coefficient ($r^2$) was 0.999 for both plasma and saliva (Figures 4 and 5).

#### *Selectivity*

Five blank samples of plasma and saliva were tested for interferences by comparison of retardation factor (RF) values obtained from samples of plasma and saliva spiked with LOFLX.

#### *Lower limits of quantification (LLOQ) and detection (LLOD)*

The lower limit of quantification (LLOQ) was evaluated by performing five replicate analyses, by the method described earlier, of plasma and saliva spiked with levofloxacin (final concentration, 1 to 16 µg/ml). LLOQ ensures that LOFLX concentrations over the entire plasma profile can be quantified, because the reported $C_{max}$ (maximum plasma concentration after an oral dose of 500 mg) of levofloxacin is 6.1 mg/L (Wagenlehner et al., 2006). A lower limit of detection (LLOD) of 1 µg/ml was determined.

#### *Stability*

The stability of LOFLX was assessed during all the storage steps and during all steps of the analytical method. No change in stability over the period of one month was observed.

**Table 1.** Extraction recovery for the assay of LOFLX in plasma and saliva (n = 5).

| Serial number | Specimens | Nominal concentration (µg/ml) | Measured concentration (mean ± SD) (µg/ml) | RSD (%) | Recovery (%) |
|---|---|---|---|---|---|
| 1 | | 2 | 1.90 ± 0.09 | 4.73 | 95.00 |
| 2 | Plasma | 4 | 3.72 ± 0.18 | 4.83 | 93.00 |
| 3 | | 8 | 7.71 ± 0.37 | 4.80 | 96.38 |
| 4 | | 2 | 1.81 ± 0.09 | 4.97 | 90.50 |
| 5 | Saliva | 4 | 3.66 ± 0.18 | 4.91 | 91.50 |
| 6 | | 8 | 7.68 ± 0.38 | 4.95 | 96.00 |

**Table 2.** Reproducibility of the analysis of LOFLX for intra-day in plasma and saliva (n = 5).

| Serial number | Specimens | Nominal concentration (µg/ml) | Measured concentration (mean ± SD) (µg/ml) | RSD (%) |
|---|---|---|---|---|
| 1 | | 2 | 1.89 ± 0.09 | 4.76 |
| 2 | Plasma | 4 | 3.71 ± 0.18 | 4.58 |
| 3 | | 8 | 7.69 ± 0.35 | 4.55 |
| 4 | | 2 | 1.88 ± 0.08 | 4.26 |
| 5 | Saliva | 4 | 3.65 ± 0.17 | 4.66 |
| 6 | | 8 | 7.66 ± 0.38 | 4.96 |

**Table 3.** Reproducibility of the analysis of LOFLX for inter-day in plasma and saliva (n = 5).

| Serial number | Specimens | Nominal concentration (µg/ml) | Measured concentration (mean ± SD) (µg/ml) | RSD (%) |
|---|---|---|---|---|
| 1 | | 2 | 1.88 ± 0.08 | 4.26 |
| 2 | Plasma | 4 | 3.71 ± 0.17 | 4.58 |
| 3 | | 8 | 7.68 ± 0.34 | 4.43 |
| 4 | | 2 | 1.88 ± 0.09 | 4.79 |
| 5 | Saliva | 4 | 3.65 ± 0.16 | 4.38 |
| 6 | | 8 | 7.65 ± 0.32 | 4.18 |

***Reproducibility***

LOFLX from spiked plasma and saliva with a standard solution of LOFLX in methanol with the same initial concentration as shown in Table 1.

***Recovery***

The known amounts of LOFLX were added to drug free plasma in concentrations ranging from 2 to 8 µg/ml. The relative recovery of LOFLX was calculated by comparing the peak areas for extracted. We assessed the precision of the method by repeated analysis of plasma and saliva specimens containing known concentrations of LOFLX. As shown in Tables 2 and 3, coefficients of variation were less than 5% in both plasma and saliva for intra-day and inter-day, which is acceptable for the routine measurement of LOFLX.

## RESULTS AND DISCUSSION

### Chromatographic characteristics

Under the chromatographic conditions described, LOFLX peaks were well resolved in plasma and saliva. The chromatograms of LOFLX in plasma and saliva are shown in the Figures 2 and 3, respectively. And it was found that the mean retention time of LOFLX was 1.382 and 1.384 in plasma and saliva, respectively. The chromatographic run time was less than 2 min.

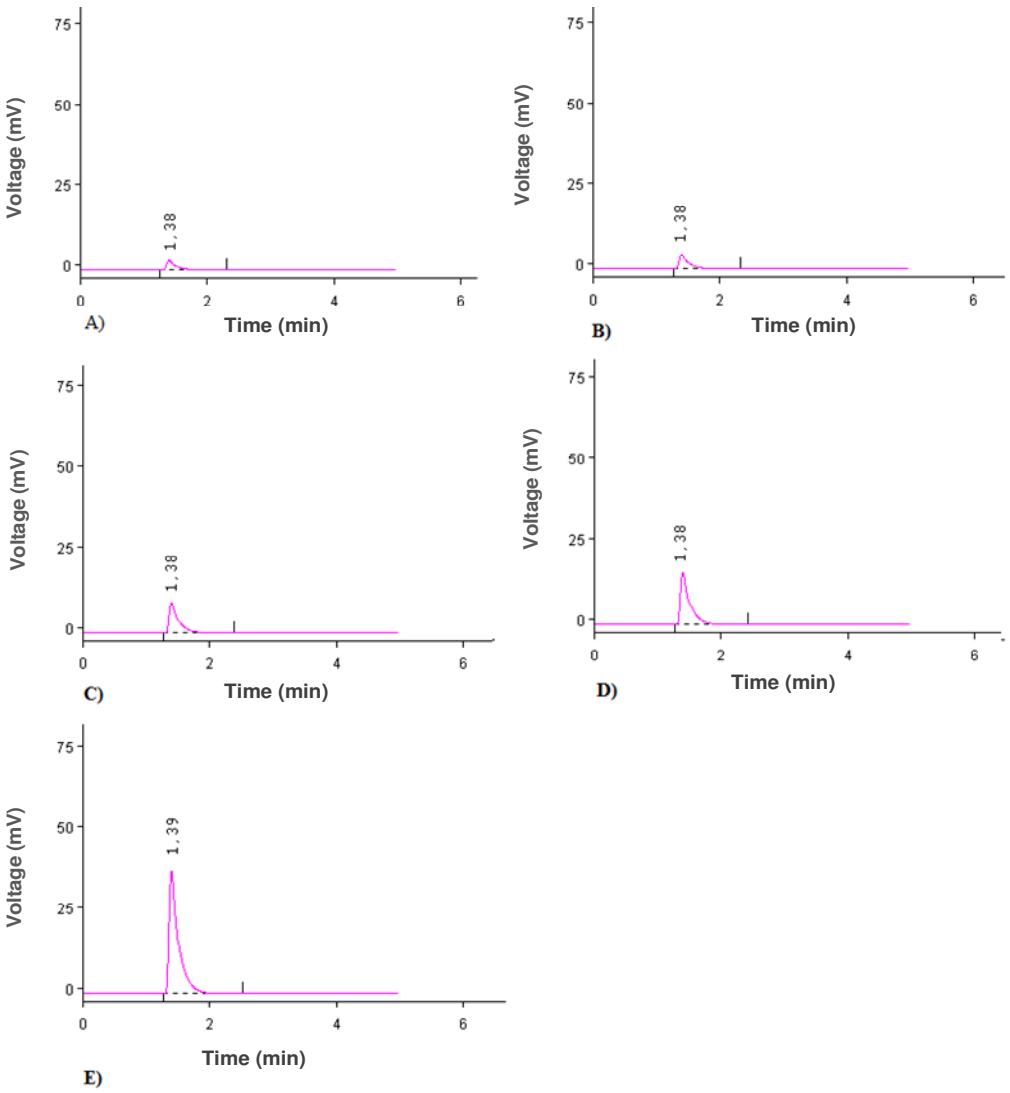


**Figure 2.** The chromatograms of LOFLX in plasma at different concentrations (1 to 16 µg/ml, A - E).

## Calibration curve

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, shown in Figures 4 and 5, respectively. The coefficients of variation for the assay were 4.83% at 4 µg/ml of the plasma concentration and 4.91% at 4 µg/ml of the saliva concentration. Hence, the sensitivity and reproducibility of our method are comparable with the most sensitive reported methods (Siewert, 2006; Djabarouti et al., 2004). The use of a smaller sample volume provides an advantage when compared with some previous methods (Bottcher et al., 2001) that require large volume of plasma samples for drug analysis. In addition, sample preparation in our method is considerably faster and easier than those in previous methods. The extraction procedure is simple and no other clean up steps are required in our method. The aim of our study was to develop a rapid and sensitive method for the routine analysis of biological samples in pharmacokinetic study of LOFLX. The respective regression equations for plasma and saliva were y = 238950.2x + 5607.514 (r = 0.999) and y = 276625.2x + 25097.29 (r = 0.999), where y is the peak-area of the drug to the internal standard, x is the concentration in plasma or saliva (µg/ml) and r is the coefficient of correlation, and that statistically confirms the linearity of this method. The limits of determination were established at 1 µg/ml in plasma and saliva, sensitive enough for drug monitoring and other purposes, such as pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of LOFLX. Blank plasma and saliva samples did not interfere with the peaks for LOFLX.

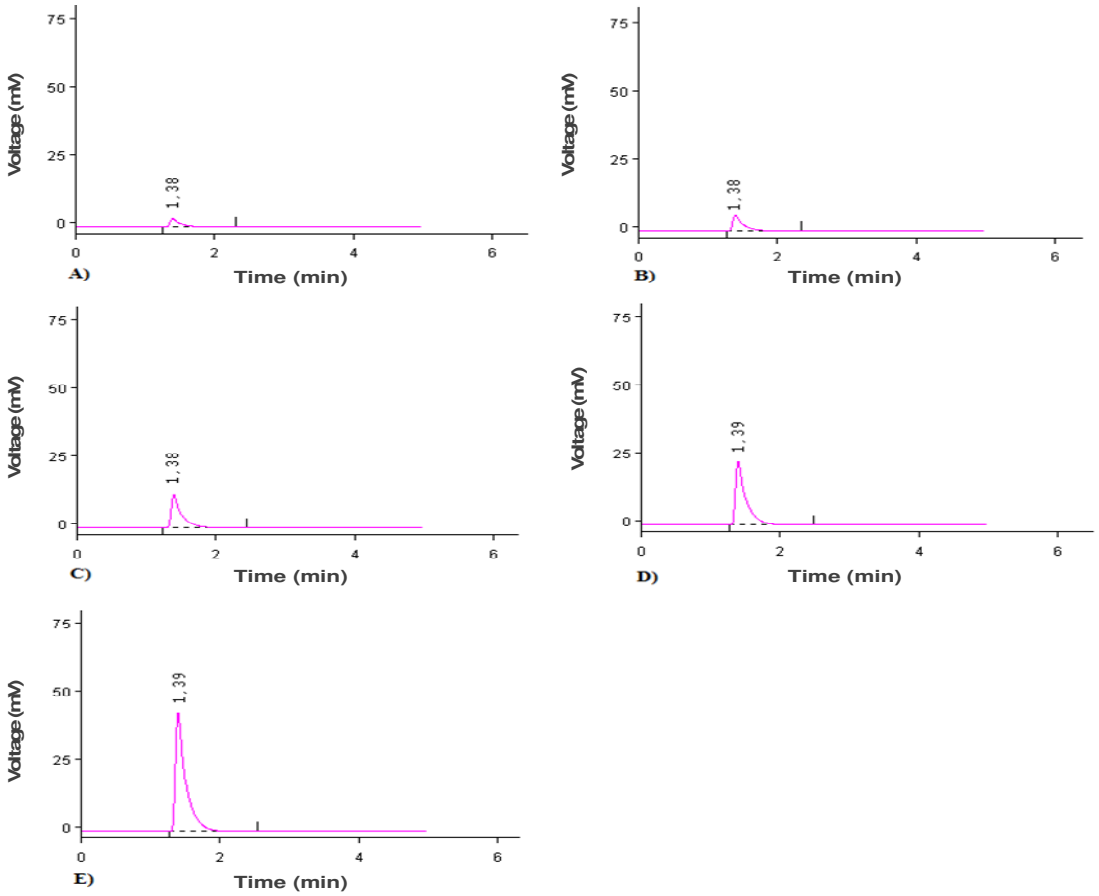


**Figure 3.** The chromatograms of LOFLX in saliva at different concentrations (1 to 16 µg/ml, A - E).

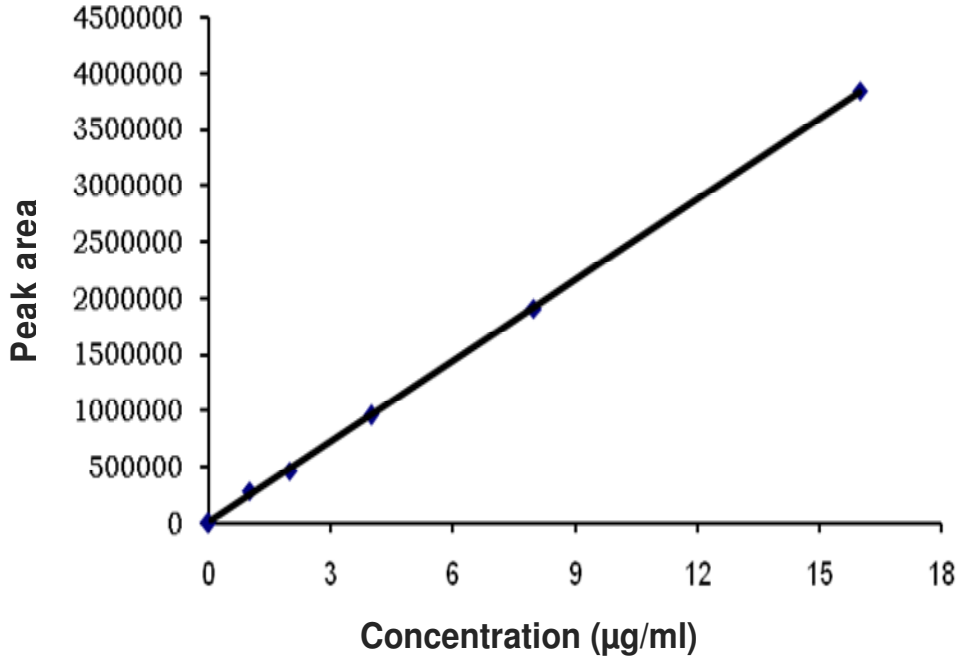


**Figure 4.** Calibration plot of LOFLX in plasma by RP-HPLC method.

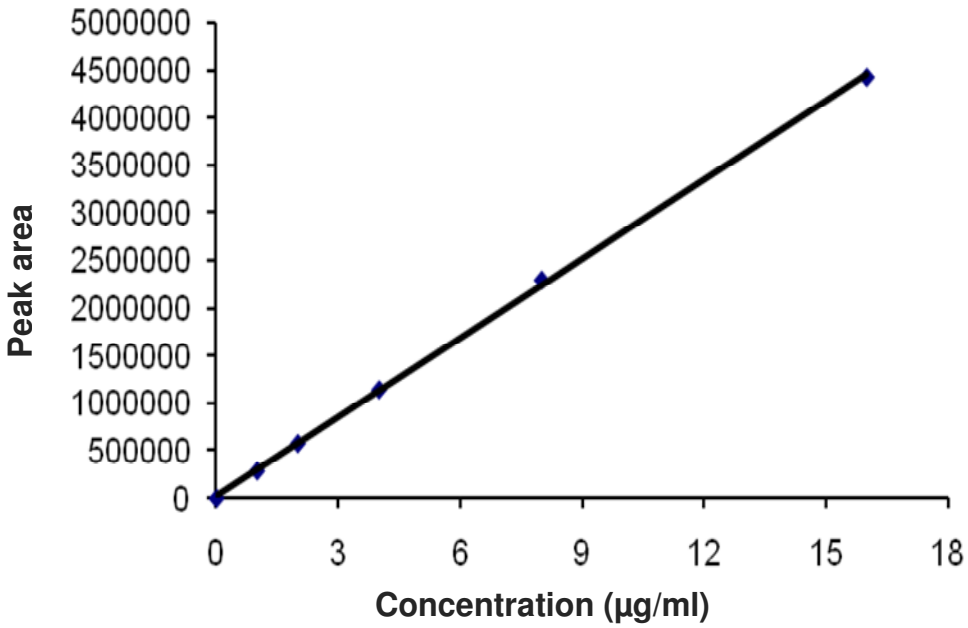


**Figure 5.** Calibration plot of LOFLX in saliva by RP-HPLC method.

The relative analytical recovery for plasma and saliva at three different concentrations of LOFLX was determined as shown in Table 1. Known amounts of LOFLX were added to drug free plasma in concentrations ranging from 2 to 8 µg/ml. The relative recovery of LOFLX was calculated by comparing the peak areas for extracted LOFLX from spiked plasma and saliva, and a standard solution of LOFLX with the same initial concentration. The average recovery was 94.79 and 92.66%, respectively in plasma and saliva (n = 5). There was high recovery in plasma of LOFLX as compared to previously reported sensitive method (Manish et al., 2011).

We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of LOFLX. As shown in Tables 2 and 3, coefficients of variation were less than 5%, which is acceptable for the routine measurement of LOFLX (Xingjie et al., 2007). This method is well suited for routine application in the clinical laboratory, because of the simple extraction procedure and good sensitivity. Over 350 plasma and saliva samples were analyzed by this method without problems, thus proving its suitability.

## Conclusion

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 (Sheikh et al., 2010). Numerous analytical studies have been reported for the determination of LOFLX in various biological fluids (Bottcher et al., 2001; Immanuel et al., 2001). The survey of these papers revealed that determination of LOFLX in saliva was not done. Therefore, the aim of this study was to determine the concentration of LOFLX not only in plasma but also in saliva so that the non-invasive therapeutic drug monitoring of LOFLX would be developed alternatively to blood analysis with particular advantage in geriatric and pediatric cases. Thus, the assay is suitable for routine assay for the determination of LOFLX in plasma and Saliva. Sample preparation is quick and cheap; therefore, the method permits the analysis of a large number of samples.

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