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Development and validation of a sensitive method for Levofloxacin in Gingival Crevicular Fluid by HPLC using UV - Visible detector

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Abstract

Increased interest in the clinical use of antibiotics for periodontal therapy required the development of a sensitive assay for the quantitation of levofloxacin in gingival crevicular fluid (GCF). The HPLC assay employs a C18 reversed-phase Hypersil BDS column with a mobile phase composed of methanol and phosphate buffer (pH 3.5). The chromatographic separation was monitored by a UV- Visible detector with an excitation wavelength of 290nm. The retention time of Levofloxacin and Ciprofloxacin were 5.55 min and 6.52min respectively. Levofloxacin was extracted from GCF collected on capillary tubes by addition of acetonitrile containing the internal standard ciprofloxacin, and phosphate buffer. The percentage mean extraction recovery of low, mid and high quality control samples was 89.53 ± 0.91 % (Mean ± SD) for Levofloxacin and it was 91.2 ± 2.2 % for Ciprofloxacin. The lowest limit of quantitation was 50 ng/ml, with a relative standard deviation of 2.56%. The interday and intraday precision at LLOQ was 3.20 ± 0.80 (mean±SD) and 3.505± 0.84 (mean±SD). The typical GCF volumes collected were 0.1-1 µl. The method was validated for the linear concentration range 50-1300 ng/ml of levofloxacin on the capillary tubes. This assay for levofloxacin was shown to be an accurate, precise and rugged method. The proposed method can be used for the estimation of Levofloxacin which was administered as in situ gels in periodontitis.

Keywords: Levofloxacin, Gingival crevicular fluid (GCF), UV- Visible detector, HPLC

1. Introduction

Periodontitis is characterized by destruction of periodontal ligament, resorption of alveolar bone and migration of junctional epithelium along the root surface. Clinical symptoms of periodontitis include changes in the morphology of gingival tissues and bleeding up on probing. In such a condition, inner layer of the gum and bone recede from the teeth and form pockets. Concerns associated with the use of systemic antibiotics encouraged development of local drug delivery systems as a viable alternative over systemic agents. Levofloxacin (OFX) is a fluoroquinolone antimicrobial agent having a high antibacterial activity against gram positive and gram negative bacteria in vitro and in vivo [1]. Chemically, ofloxacin, a fluorinated carboxyquinolone, is the racemate, (±)-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 [2] (Fig. 1).

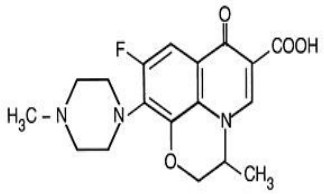

Figure 1: Chemical structure of Levofloxacin

The mechanism of the activity is based on the inhibition of the DNA-gyrase of the bacteria. In vitro studies had shown that OFX has early bactericidal activity against *Mycobatcerium tuberculosis* [3]. Due to the low flow rate of GCF, limited volumes are obtained (0.1-lµl) thus assays measuring levels of drugs in this fluid must have a greater sensitivity than assays measuring drugs in serum or plasma [4-6]. Samples were collected from patients with periodontal disease using capillary tubes.

Several high-performance liquid chromatography (HPLC) methods have been developed for measuring OFX concentration in body fluids, but literature survey revealed that no method was available for the determination of OFX in GCF [7-13]. Thus, the aim of this study was to develop and validate a simple and rapid HPLC assay for measuring OFX levels in gingival crevicular fluid that is free of interference. With lower limit of quantitation (LLOQ) of 50 ng/ml of levofloxacin on the capillary tubes with the excellent precision and accuracy.

2. Experimental

2.1. Materials and reagents

Levofloxacin was obtained as gift sample from Microlabs (Bangalore). Ciprofloxacin was obtained as gift sample from Microlabs (Bangalore) used as internal standard. Methanol, acetonitrile were supplied by Qualigen fine chemical (HPLC or ACS grade) and used for the mobile phase preparation and as diluents respectively. Disodium hydrogen phosphate, sodium dihydrogen phosphate and Ortho Phosphoric acid AR grade were supplied by S.D. Fine Chemicals (Mumbai), used for the mobile phase preparation. Standard solutions

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were prepared using levofloxacin in acetonitrile and phosphate buffer. Drug free GCF and GCF containing drug were supplied by Government dental Hospital (Bangalore). Microcenterifuge tubes (Tarsons, Mumbai) (1.5 ml) used to spike the standard and QC samples.

2.2. Apparatus

A Shimadzu liquid chromatograph equipped with a model LC-10ADvp gradient pump, Rheodyne 7725*i* injector with 20 µl loop and model SPD-M10Avp UV-Visible detector was used for the analysis. The separation was performed on a Hypersil BDS C_{18} (250 mm x 46 mm I.D.) column (Thermo, USA), with 5 µm particle diameter.

2.3. Preparation of solutions

2.3.1. Preparation of the internal standard solution

A 10 µl ml^{-1} ciprlevofloxacin internal standard solution was accurately prepared in HPLC grade acetonitrile and phosphate buffer.

2.3.2. Preparation of Standard solution

A 10 µg ml^{-1} levofloxacin reference standard solution was accurately prepared in HPLC grade Acetonitrile and phosphate buffer (levofloxacin stock).

2.3.3. Clinical sample collection

Samples were collected from patients with periodontal disease using capillary tubes. Extreme care was taken to minimize GCF contamination with tooth surface debris. To minimize that the tooth surface above the periodontal pocket collection site was wiped with a cotton swab to remove any debris. The GCF fluid flowed from the gingival pocket up the capillary tubes by capillary action. The lower portion of the capillary tube was placed between the jaws of the Periotron and a score was obtained. Sample volumes were determined by interpolation from a volume versus Periotron score calibration curve. GCF volumes were used to report levofloxacin as concentration per unit volume. Typical volumes obtained were between 0.1-1.0 µl.

2.4. Extraction procedure

Samples were examined to verify the capillary tubes were at the bottom of the microcentrifuge tubes. The GCF standard and GCF samples were extracted using Ciprofloxacin internal standard solution (20 µl) and Acetonitrile (1 ml) were added to each tube. Samples were vortexed for 30sec and centrifuged for 5 min at 4000 rpm. The supernatant liquid was taken in a 100 µl autosampler vials and injected into the HPLC.

2.4.1. GCF standard curve

GCF standard solutions were prepared using 10, 20 and 30 µg ml^{-1}concentration of Levofloxacin from levofloxacin stock into that GCF, internal standard Ciprofloxacin, acetonitrile were added respectively.

2.4.2 Quality control (QC) samples

QC samples were made from different time intervals of GCF samples- 0hr, 1hr, 2hrs, 4hrs, 8hrs, 16hrs, 4 days, 8days, 14days and 30 days respectively. The each time interval samples are spiked separately into 100 µl of acetonitrile from that 60µl was taken into a 1.5 ml micro centrifuge tubes followed by 20µl of internal standard ciprofloxacin, 80µl of phosphate buffer and 1ml of acetonitrile were added respectively. The prepared samples were vortexed for 30sec and centrifuged for 5 min at 4000 rpm. The supernatant solution was taken in a 100 µl autosampler vials and injected into the HPLC.

2.5. HPLC conditions

The mobile phase consisted of methanol and a phosphate buffer containing 20mM disodium hydrogen phosphate and 20mM of sodium dihydrogen phosphate pH 3.5 adjusted with phosphoric acid (32:68 v/v) at a flow rate of 1 ml/min. The mobile phase was filtered through a 0.45 µ membrane filter (Sartorius, Germany) and degassed before analysis. The injection volumes were 20 µl and the run time was 15 min. The UV-Visible detector has an excitation wavelength of 290 nm.

3. Results and discussion

Three quality control pools were prepared LQC (QC1), MQC (QC2) and HQC (QC3) in pre-screened human serum and 1µl was spiked to validate this method for OFX in GCF. All quality control samples and standard curve samples were prepared using human serum instead of GCF because GCF was not commercially available not easily collectible.

3.1. Development of Chromatogram

The chromatogram of sample containing only levofloxacin in figure 2 showed only one major peak at 5.55 min. The chromatogram of a GCF sample of levofloxacin is shown in Fig. 2. The retention time of levofloxacin and ciprofloxacin was 5.55 and 6.52 min respectively.

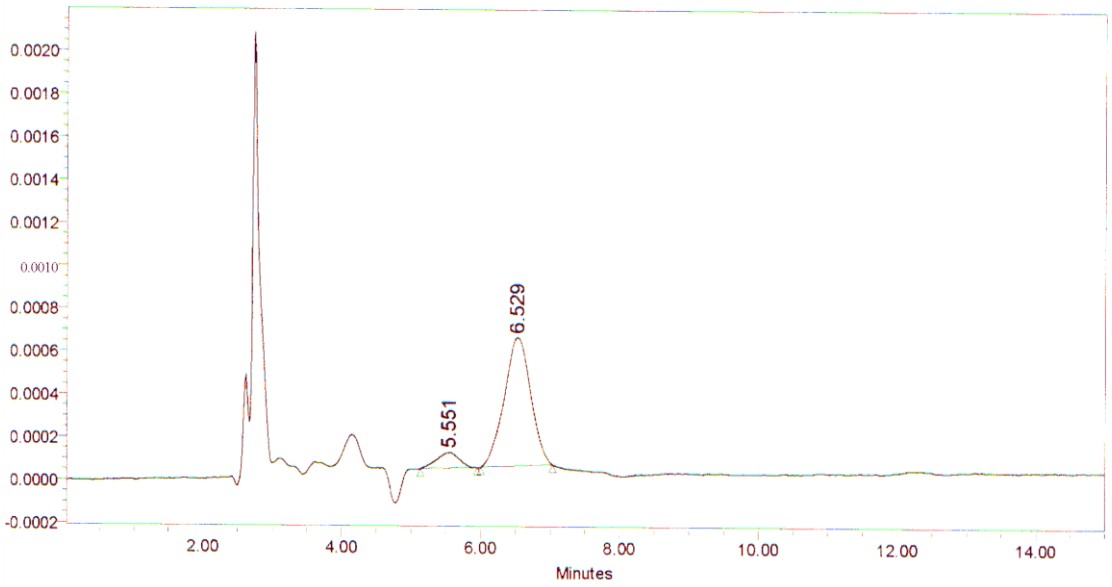

Figure 2: Chromatogram showing levofloxacin and ciprofloxacin (IS) in GCF

3.2. Linearity

The linear range for OFX in human serum was validated using 6 standards in triplicate covering the range 50-1300 ng/ml of OFX. The lowest limit of quantitation was 50 ng/ml, with a relative standard deviation of 2.56%. Standard curves were generated using a weighted (l/x) linear least squares regression of the OFX peak area vs. concentration.

Table 1 summarizes the correlation coefficients, slope, intercept and the pooled percent errors for all validation standard curves. The data demonstrates the linearity and the reproducibility of the standard curves for the developed method.

Table 1: Results for summary of standard curve

Parameter	Value
Correlation Co-efficient (r^2)	0.9978
Slop	0.5773
Y-intercept	+ 0.0421
%RSD	2.56%

3.3. Precision and accuracy

The interday and intraday precision at LLOQ was 3.20 ± 0.80 (mean±SD) and 3.505± 0.84(mean±SD). Table 2 summarizes the precision and accuracy results for the method. The precision (%R.S.D., relative standard deviation: (S.D./mean)x 100) for QC1, QC2, QC3 concentrations were 8.36%, 1.25%, 0.58%, respectively. The accuracy (%REC, relative recoveries: (back calculated value/nominal value)x 100) for QC1 QC2 and QC3 were within ± 1.00%. The accuracy of the method varies from 99.0 to 100.5% among all batches.

Table 2: Results for Precision and accuracy of QC samples

Conc.	Drug		IS area		Area ratio		Pred. Conc.	% Bias	% REC
	Avg. area (n=6)	%RSD	Avg. Area (n=6)	%RSD	Avg.	%RSD			
0.20	12975	8.6136	78949	0.5346	0.1643	8.3600	0.1982	-0.85228	99.10
0.80	40301	1.2556	77708	0.5927	0.5186	1.7355	0.8029	0.372871	100.36
1.60	75104	0.5803	76261	0.7617	0.9848	0.4459	1.5987	-0.0799	99.91

3.4. Extraction Recovery

The recovery was measured over the linear range of 1-25 µg/ml of OFX by comparing a triplicate standard curve prepared in acetonitrile to a triplicate standard curve prepared in spiked serum. The results are shown in Table 3 and the percent extraction recovery for QC1, QC2, and QC3 was 87.92 %, 88.89 % and 90.76 % respectively. The percentage mean extraction recovery of low, mid and high quality control samples was 89.53 ± 0.91 % (Mean ± SD) for Levofloxacin and it was 91.2 ± 2.2 % for Ciprofloxacin.

Table 3: Results for Recovery study

	Unextracted drug Avg. area (n=3)	Extracted drug Avg. area (n=3)	% Extraction recovery
QC1	64501.67	60280	93.45
QC2	279770	253538.3	90.73
QC3	1440640	1364623	94.72

3.5. Specificity

The specificity of the method was demonstrated by running chromatograms of drug free human serum sample. All chromatograms demonstrated no peaks which would interfere with the OFX or internal standard peaks.

3.6. Stability study

The method was also validated for its stability. The processed samples in the acetonitrile-buffer were stable at least 20 h at room temperature. The GCF samples were stable at least 3 weeks at -20°C with three thaw and freeze cycles. The mean nominal concentration of three freeze thaw cycles, short term stability (Sample processing time) and long term stability (-20° C for 2 weeks) were 95.6 ± 0.28 %, 94.05 ± 0.21% and 96.2 ± 0.42% respectively.

3.7 *Selectivity*

The selectivity of the method was performed by spiking the concentration of LLOQ at six different aliquots plasma samples and the regression equation was used for the quantitation of unknown samples.

4. Conclusion

The validated method for OFX in GCF described in this paper is precise, accurate and rugged and was shown to be sensitive. This method is suitable for use as the standard method for analysis of OFX in GCF for future products such as insitu gels and product modifications for the treatment of periodontal disease.

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