A Single Reversed-Phase UPLC Method for Quantification of Levofloxacin in Aqueous Humour and Pharmaceutical Dosage Forms

Himanshu Gupta[1,*], M. Aqil[1,*], R.K. Khar[1], Asgar Ali[1], and **Prakash Chander[2,*]**

[1]Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), New Delhi-110062, India and [2]Waters India Pvt. Ltd., A-41, Sector 62, Noida, India

Abstract

An attempt was made to develop a single, rapid, specific, and sensitive gradient reversed-phase ultra-performance liquid chromatographic method for quantitative analysis of levofloxacin. The single method thus developed is applied for the quantification of levofloxacin both in aqueous humour as well as pharmaceutical dosage forms (i.e., tablets and eye drops). The newly developed method is applicable for pharmacokinetic studies of eye formulations. The chromatographic separation of levofloxacin was achieved on a Waters Acquity HSS T-3 column (100 × 2.1 mm, 1.8 µm) within a short run-time of 5 min. The method was validated according to the ICH guidelines with respect to system suitability, linearity, limit of quantitation and detection, precision, accuracy, robustness, and specificity. Forced degradation studies were also performed in levofloxacin bulk drug samples to demonstrate the stability-indicating power of the developed ultra-performance liquid chromatography method. The developed method was then successfully applied for the ocular pharmacokinetic study of levofloxacin eye formulations and assay of levofloxacin pharmaceutical dosage form.

Introduction

Levofloxacin is the active L-isomer of ofloxacin, a fourth-generation fluoroquinolone derivative (Figure 1). Levofloxacin is used to treat external infections of the eye, such as acute and subacute conjunctivitis, bacterial keratitis, and keratoconjunctivitis. It has broad spectrum antimicrobial activity against gram-positive and gram-negative species. Levofloxacin is found to be more effective than ciprofloxacin both in vitro (1) and in vivo activity (2). Levofloxacin is available in solid oral dosage form, oral solution, ophthalmic solution, and intravenous preparation. A 0.5% and 1.5% ophthalmic solution of levofloxacin is available in the market as eye drops for the treatment of corneal ulcer and bacterial eye infections caused by susceptible strains Corynebacterium species, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, Viridans group streptococci, *Pseudomonas aeruginosa*, and *Serratia marcescens*, etc. (3).

Delivery to ocular region is a challenging task. Only 1–2% of total instilled drug is able to reach the site of action due to eye physiological barriers. Several new preparations have been developed to prolong the contact time of the medicament on the ocular surface and to slow down drug elimination, such as inserts (4), collagen shields (5), hydrogel, and nanoparticles (6). Efficacy of these novel formulations were evaluated on the basis of the amount of drug permeated to the anterior chamber (i.e., aqueous humour). A minute quantity of the drug is able to reach in the aqueous humour, which is generally difficult to quantify and requires highly sensitive and advanced techniques.

A detailed literature search reveals many analytical procedures for determination of levofloxacin by spectrophotometry (7), potentiometry, conductometry (8), and liquid chromatography (9–11). There are some analytical methods, which had been published describing the high-performance liquid chromatography (HPLC) assay for levofloxacin in injectable formulations (12,13). Some HPLC methods for the estimation of levofloxacin in human plasma and urine were also reported (14–17). Most of the reported methods involve a troublesome mobile phase (buffers) and advanced detection methods (fluorescence or mass detectors) (18). Recently, Dae-Jim et al. (19) reported an ultra-performance liquid chromatography (UPLC) method for quantification of levofloxacin in human plasma, but we have not found any reported literature for quantification of levofloxacin in aqueous humour and pharmaceutical formulations. Literature and data further demonstrates that there is no selective difference in composition of aqueous humour of different species, such as dogs,

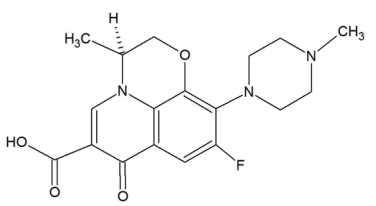

Figure 1. Structure of levofloxacin.

* Author to whom correspondence should be addressed: email himanshu18in@yahoo.com, aqilmalik@yahoo.com, prakash_chander@waters.com.

monkeys, rabbits, and humans (20). Hence, the developed method for quantification of levofloxacin in aqueous humour of rabbits can also be used for aqueous humour samples of humans and other specimens.

In our present work, an attempt was made to develop a single, sensitive, simple, and reliable UPLC method for quantification of levofloxacin in aqueous humour and pharmaceutical dosage forms. The developed method was thus applied for ocular pharmacokinetic study and assay determination of dosage forms.

Experimental

Materials and reagents

The drug levofloxacin was received as a gift from Micro Labs, Ltd. (98.91% pure on dried basis) (Karnataka, India). HPLC-grade acetonitrile was purchased from Qualigens Chemical (Mumbai, India). Trifluoro acetic acid (TFA) was purchased from Merck (Mumbai, India). To prepare solutions, ultra-pure water was supplied by a Millipore Milli-Q purification unit (Billerica, MA). All other reagents used are analytical grade unless otherwise mentioned.

Apparatus

The chromatographic equipment consisted of Waters Acquity system equipped with binary solvent delivery pump, an autosampler, a thermostatic column compartment, and tunable UV detector. The instrument is equipped with a Waters Empower software package for data acquisition and handling. A Waters Acquity HSS T-3 C_{18} column (100 × 2.1 mm i.d., 1.8 µm) maintained at 50°C was used for the analysis.

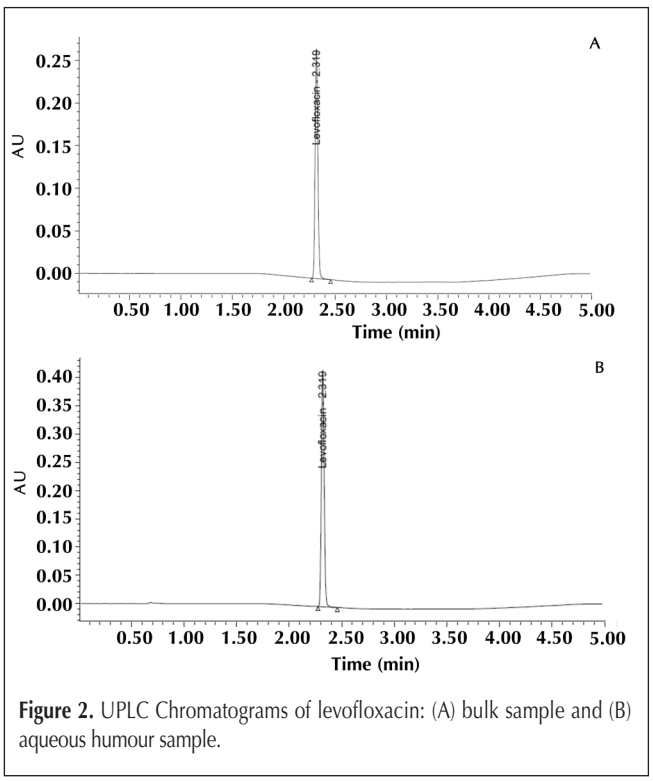

Figure 2. UPLC Chromatograms of levofloxacin: (A) bulk sample and (B) aqueous humour sample.

Chromatographic conditions

The mobile phase was consisted of a mixture of A: 0.1% aqueous TFA and B: acetonitrile, delivered at a constant flow rate of 0.45 mL/min. Separation was performed under gradient programme T (min)/%B: 0/8, 1/8, 2/20, 3/20, 4/8, and 5/8. Column temperature was fixed at 50°C, whereas sample temperature was 5°C. The monitoring wavelength was 288 nm, and the injection volume was 2 µL. All mobile phases were filtered through a 0.22-µm Millipore filter.

Standards and samples preparation

Primary stock solution of levofloxacin bulk drug (250 µg/mL) was prepared by dissolving appropriate amount of levofloxacin in diluent (acetonitrile). These solutions were further diluted to obtain working standard solutions (20–45 µg/mL) in duplicate.

For aqueous humor samples, whole eyeballs of rabbits were procured from a local slaughter house and transported to the lab in ice. Aqueous humour was collected by puncturing the anterior chamber of the eye and storing samples at +4°C until analysis. The cornea were then carefully removed along with 5–6 mm scleral tissue of the eye and stored in normal saline until further use. For aqueous humour, no matrix effect was observed due to the low amount of protein content (21). A 0.45-µL aqueous humour sample was injected directly onto the column without any preliminary treatment. Weekly injection of an aqueous humour sample and bulk drug sample showed similar chromatograms with no change in the concentration of levofloxacin for at least one month when the solution was stored at +4°C.

Method Validation

Suitability

The suitability of the mobile phase was decided on the basis of the sensitivity of the assay, suitability for stability studies, time required for the analysis, ease of preparation, and use of readily available and cost-effective solvents. The newly developed UPLC method was validated according to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (22–25). The method was validated with respect to linearity, limit of detection and quantitation, precision, accuracy, robustness, and specificity (26).

Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations of the analyte in the sample. A regression line was fitted on the back-calculated concentrations by applying the selected regression model. Seven concentration levels ranging from 20 to 45 µg/mL were analyzed in duplicate.

Table I. Properties of Developed UPLC Method

System	Elution time (min)	Flow rate (mL/min)	Injection volume (µL)	Run-time (min)	Tailing factor	USP plate count
UPLC	2.319	0.45	2	5	1.10	35520

Limits of detection and quantitation (Trueness)

The limits of detection (LOD) and quantitation (LOQ) were estimated by injecting a series of dilute solutions with known concentration by visual evaluation and signal-to-noise ratio.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample (independent assays). Assay method precision was carried out using six independent test solutions and a standard preparation of same concentration. The intermediate precision of the assay method was also evaluated using different analyst on three different days. Variance of repeatability and intermediate precision were computed from estimated concentrations, and precision was expressed by relative standard deviation (RSD) at each level.

Accuracy

Accuracy of the developed method was determined by conducting recovery experiments. The accuracy of the assay method was evaluated in triplicate using three concentration levels 80% (24 µg/mL), 100% (30 µg/mL), and 120% (36 µg/mL).

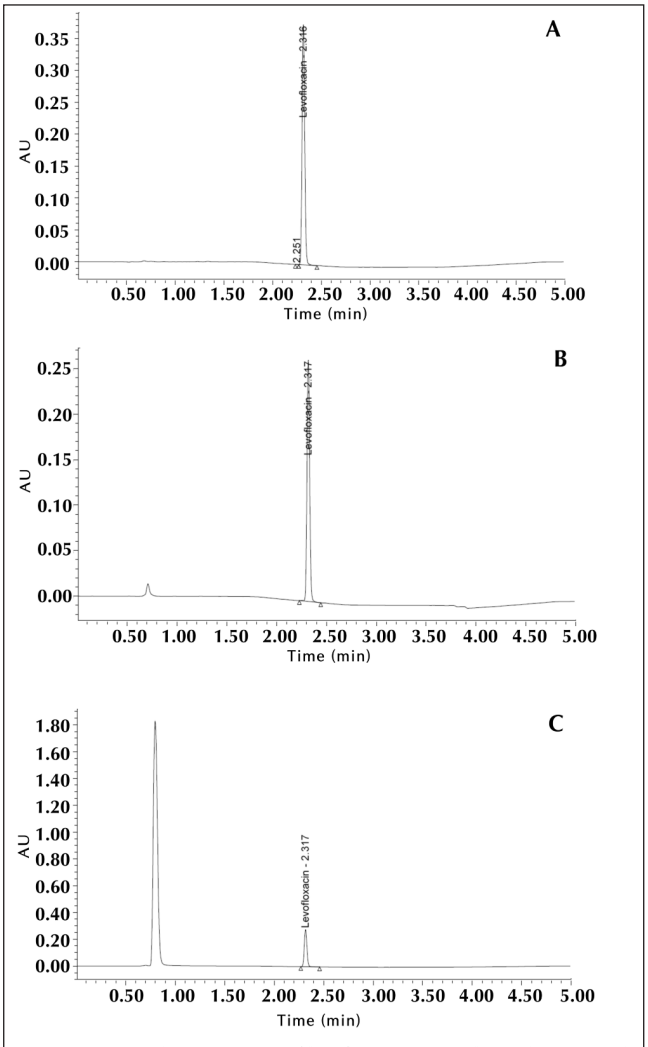

Figure 3. UPLC chromatograms of levofloxacin stressed samples: (A) acid degeneration, (B) oxidative degeneration, and (C) reductive degeneration.

Robustness

To determine the robustness of the method, experimental conditions were purposely altered and examined by injecting system suitability solution. The flow rate varied by ±0.05 mL/min (0.40 and 0.50 mL/min). Column temperature varied by ±2°C (48°C and 52°C). The wavelength number was varied by ±2 nm (286 nm and 290 nm).

Stress studies

Forced degradation studies were also performed on bulk drug samples using the following conditions: acid hydrolysis (0.1 N hydrochloric acid), base hydrolysis (0.1 N sodium hydroxide), heat (105°C for 96 h), photolytic (UV and sunlight for 96 h), oxidation (30% hydrogen peroxide), and reduction (10% sodium metabisulphite). Peak purity test was carried out for levofloxacin peak by using photodiode array (PDA) detector in stress samples.

Application of the Developed Method

Ex vivo ocular pharmacokinetic study

Ex vivo drug release studies were performed in triplicate with some modification as described earlier (27). Isolated rabbit corneas were used to study the ocular pharmacokinetics of developed levofloxacin in situ gel formulation and marketed eye drops. The study was carried out in a modified franz diffusion chamber. The upper chamber served as a donor compartment in which 100 µL of drug solution/formulation was placed. Freshly excised rabbit cornea was fixed between the clamped donor and receptor compartments of Franz diffusion cell in such a way that its epithelial surface faced the donor compartment. The lower chamber, which served as a receiver compartment, was infused with the fresh aqueous humour of rabbit. The whole system was maintained at 37 ± 0.5°C. One milliliter of perfusate was collected in preweighed microcentrifuge tubes at periodic time intervals up to 4 h and replaced with fresh aqueous humour to maintain sink condition. The collected samples were then subjected to the quantification of levofloxacin using the developed UPLC procedure.

Estimation from pharmaceutical dosage forms

Assay was performed on two different marketed formulations: tablets and eyedrops. Twenty tablets were weighed and powdered. Accurately weighed quantity of tablet powder equivalent to about 25 mg of levofloxacin was transferred to a 100-mL volumetric flask. A few milliliters of acetonitrile were then added and

Table II. Results of Regression Analysis of Linearity Data of Levofloxacin Bulk Drug

Parameters	Results
Range (µg/mL)	20–45
Slope	24296.58
Intercept	–217292
R^2	0.999
LOD (µg/mL)	0.015 µg/mL
LOQ (µg/mL)	0.05 µg/mL

sonicated before the volume was adjusted with Milli-Q water. The solution was then appropriately diluted to get a final concentration of 30 µg/mL. For the assay of eyedrops, 0.5 mL of eyedrops was diluted to 100 mL in volumetric flask with Milli-Q water. Final solutions were filtered through 0.22-µm filter. 0.45 µL of these sample preparations were injected into the column and analyzed in triplicate.

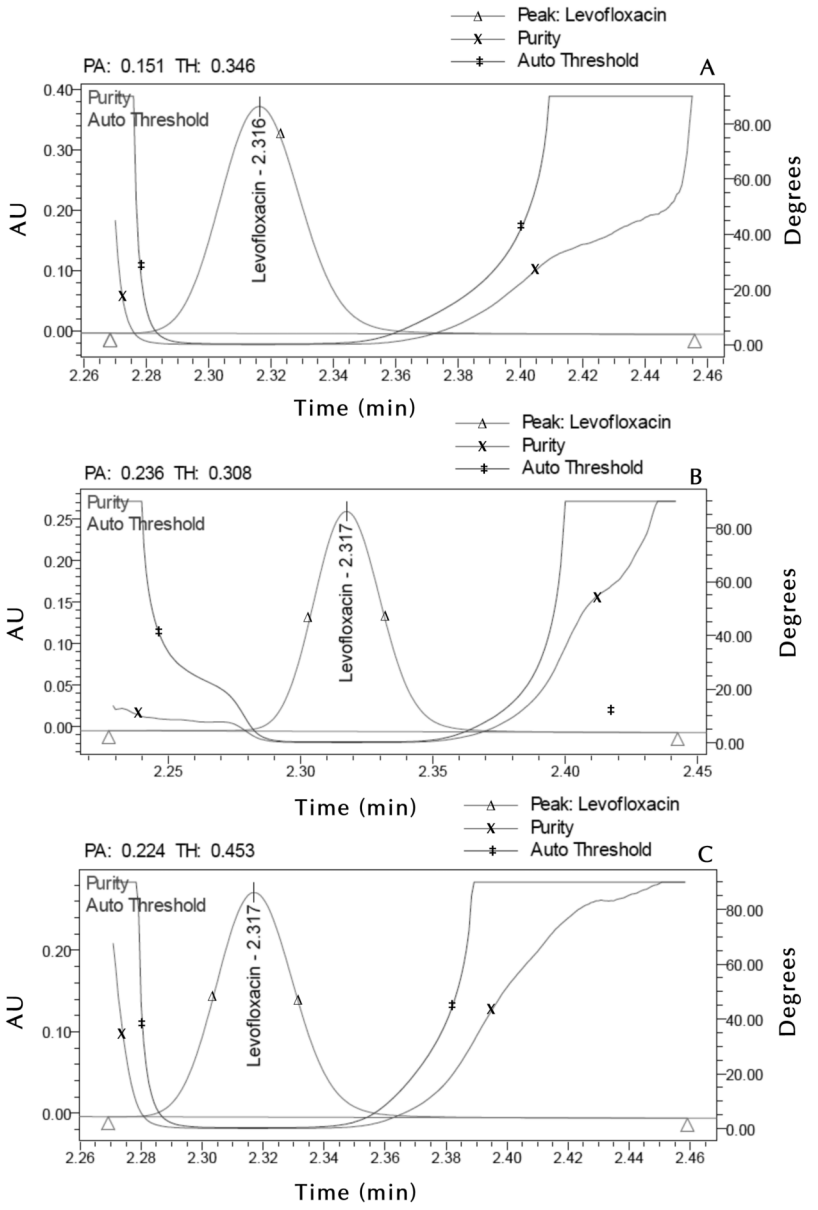

Figure 4. PDA peak purity: (A) acid degeneration, (B) oxidative degeneration, and (C) reductive degeneration.

Table III. Intermediate Precision and Repeatability Data

	Intermediate precision				Repeatability	
	Inter-day measured conc.		Different analyst measured conc.		Intra-day measured conc.	
Levels	Average conc. recovered (µg/mL)	% RSD	Average conc. recovered (µg/mL)	% RSD	Average conc. recovered (µg/mL)	RSD%
24	22.13	1.59	23.87	1.36	23.34	0.96
30	30.11	1.19	29.80	1.01	29.24	0.94
36	35.89	0.70	34.63	1. 36	34.86	1.42

Results and Discussions

Method development by UPLC

The main target of the chromatographic method was to achieve separation and quantification of the main component levofloxacin from the aqueous humour, bulk drug, and pharemaceutical formulation. Gradient system is always preferred over isocratic system in order to achieve improved peak shape and resolution. Sometimes in the isocratic system, peak is eluted late so gradient system was used to reduce runtime. Hence, it was decided to use gradient UPLC mode under gradient programme T (min)/%B: 0/8, 1/8, 2/20, 3/20, 4/8, 5/8. The injection volume in UPLC was set to 2 µL. The mobile phase containing a mixture of 0.1% aqueous TFA and acetonitrile with the flow rate of 0.45 mL/min was used. The response of levofloxacin was found to be adequate at 288 nm. Primary a BEH C_{18} column (100 × 2.1 mm, 1.7 µm) was used during development process. Obtained chromatogram shows tailing in the levofloxacin peak, which is not acceptable. USP plate counts were also low: ~ 3000. The BEH C_{18} column was then replaced with an Acquity HSS T-3 column (100 × 2.1 mm, 1.8 µm). The peak found was sharp and acceptable with a USP plate count of > 35,000 (Table I). Using these conditions, a satisfactory peak of levofloxacin eluting around 2.319 min was obtained with a total runtime of 5 min (Figure 2A). Identification of levofloxacin was confirmed by comparison of its UV spectrum with the levofloxacin standard. Graphs were plotted between the mean peak area of the drug with respect to concentration. The curves were found to be linear in the concentration range of 20–45 µg/mL of levofloxacin.

UPLC method validation

The aim of the validation study was to confirm the method suitability for its intended purpose of routine analysis. We have obtained similar chromatograms from bulk samples and aqueous humour samples of levofloxacin, incorcordant with previously reported literature (Figure 2B) (21). Hence, this method can be used for determination of levofloxacin in aqueous humour as well as dosage forms. The method validation was carried out on this single method with acetonitrile as diluent. The assay values of different aqueous humour samples,

bulk drug, and pharmaceutical preparation were found to be in the range of 80–120%. Forced degradation studies were also performed on levofloxacin bulk drug samples to demonstrate the stability-indicating power of the newly developed UPLC method.

System suitability

System suitability test were used to verify the reproducibility of the proposed method to produce good resolution peaks of interest each time. System suitability is analyzed in terms of tailing factor (must be < 1.5), theoretical plate counts (should be > 20,000), retention time, etc. The result for proposed UPLC method is given in Table I. According to the results presented, the proposed UPLC method fulfills these requirements within the accepted limits.

Linearity

For linearity, seven concentration levels ranging 70–130% (v/v) for levofloxacin in triplicate were used. Regression equation obtained was:

$$y = 24{,}297x - 217{,}292$$

with the correlation coefficient (R^2) greater than 0.999. The relationship between peak area and concentration was linear in the calibration range. Results obtained from regression analysis of the linearity data for levofloxacin is summarized in Table II.

LOQ and LOD

LOQ was determined by a signal-to-noise ratio of ≥ 10, whereas LOD was determined at a signal-to-noise ratio of ≥ 3. LOQ and LOD values for levofloxacin were found to be 0.05 µg/mL and 0.015 µg/mL with a signal-to-noise ratio of 12 and 7, respectively (Table II).

Precision

The precision of the developed method was evaluated as repeatability. Intermediate precision was evaluated by carrying out six independent assays at 24, 30, and 36 µg/mL concentrations of levofloxacin on different days by different analysts. For repeatability the %RSD of assay of levofloxacin was found to be in a range of 0.94–1.42, whereas the %RSD of the results obtained in intermediate precision study was in a range of 0.70–1.59 (Table III). These %RSD values are well within the generally acceptable limit of 2%, confirming good precision of the assay method.

Accuracy

The accuracy of the developed method was determined by the recovery experiments. Results obtained from recovery studies are given in Table IV. The percent recovery of levofloxacin in these samples ranged from 98.7 to 100.9% with %RSD of values of 0.52, 1.11, and 1.55% at 80, 100, and 120%, respectively (Table IV). High recovery results obtained from the proposed UPLC assay method indicates that this method can be used for routine quantitative quality control analysis of pharmaceutical dosage form.

Robustness

To prove the reliability of the analytical method during normal usage, some small but deliberate changes were made in analytical method, such as flow rate, column temperature, and UV detector wavelength. Changes in chromatographic parameters, such as theoretical plates and tailing factor, were evaluated for the analysis. In all the varied chromatographic conditions, the chromatogram for system suitability solution showed satisfactory resolution (%RSD < 2%) with no significant changes in chromatographic parameters (Table V).

Table IV. Recovery Analysis of Levofloxacin

Concentration	Actual (µg/mL)	Recovered (µg/mL)	Recovery (%)	RSD (%)
80% level of	22.1	21.92	99.2	0.52
test conc.	21.2	20.81	98.2	
	21.3	21.06	98.9	
100% level of	27.1	27.11	100.1	1.11
test conc.	28.3	27.95	98.7	
	29.7	29.97	100.9	
120% level of	38.5	39.06	101.4	1.55
test conc.	37.5	36.90	98.4	
	40.5	40.74	100.6	

Table V. Robustness Evaluation of the Developed UPLC Method

	Levofloxacin*		
Chromatographic changes	%RSD (Peak area) $n = 6$	Tailing factor	Theoretical plate count
Flow rate (mL/min)			
0.40	0.3%	1.12	35410
0.45†	0.5%	1.10	35520
0.50	0.4%	1.11	35780
Temperature (°C)			
48	0.4%	1.11	33953
50†	0.5%	1.10	35520
52	0.4%	1.14	34648
UV wavelength (nm)			
286	0.5%	1.12	34269
288†	0.3%	1.10	35520
290	0.3%	1.15	34962

* All values given are taken as mean of six individual values.
† Optimum condition.

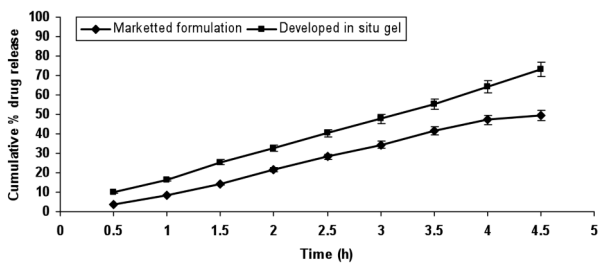

Figure 5. Ex vivo drug release profile of ocular formulations. Values are expressed as mean ± standard deviation ($n = 5$).

Stress study

Stress studies of the drug APIs is utilized for the validation of the stability-indicating analytical procedures. It is the ability of analytical method to measure the analyte response in the presence of its degradents. The results obtained from the forced degradation studies are summarized in Table VI. During the forced degradation study, a considerable degradation of drug substance was observed in acidic, oxidative, and reductive conditions (Figure 3). The chromatograms were checked for the appearance of any extra peak. Peak purity of these samples under stressed conditions was verified using a PDA detector (Figure 4). The purity of the principle and other chromatographic peaks was found to be satisfactory. This study confirmed the stability indicating power of the UPLC method.

Table VI. Summary of Forced Degradation Studies

Stress condition	Time	Purity angle	Purity threshold
Acid hydrolysis (1 N HCl at RT)	12 h	0.151	0.346
Remark: Very minute degradation was observed, but main peak is homogeneous			
Base hydrolysis (1 N NaOH at RT)	12 h	0.128	0.294
Remark: No degradation. Main peak is homogeneous.			
Oxidation (30% H_2O_2 at RT)	12 h	0.242	0.263
Remark: Degradation was observed, but the main peak is homogenous.			
Reduction (10% $Na_2S_2O_5$)	12 h	0.559	0.855
Remark: Degradation was observed, but the main peak is homogenous. Degraded product was eluted separately.			
Thermal (105°C)	24 h	0.178	0.255
	48 h	0.190	0.257
	72 h	0.198	0.266
Remark: No degradation was found.			
Sunlight	24 h	0.193	0.256
	48 h	0.194	0.257
	72 h	0.206	0.260
Remark: No degradation was found.			

Table VII. Formula of the Developed In Situ Formulation

Ingredients	Concentration (w/v)
Levofloxacin	0.5%
Chitosan	0.5%
HPMC	0.5%
NaCl	0.45%
Methyl Paraben	0.1%
Water (q.s.)	100%

Table VIII. Application of UPLC Method to the Determination of Levofloxacin from Pharmaceutical Dosage Forms

Commercial formulations	Labelled claim (mg)	Mean amount found	Number of samples	SD of the amount found	RSD % of the amount found	Recovery (%)
Levoquine (500 tablets)	500 mg	501.97 (mg/tablet)	6	1.3946	0.28	100.4%
Leeflox eye drop (0.5% w/v)	5 mg/mL	5.02 (mg/mL)	6	0.0695	1.37	100.4%

Application of the Developed Method

Ex vivo ocular pharmacokinetic study

Ex vivo drug release profile of the marketed formulation and developed in situ formulation were determined using fresh aqueous humour collected from isolated rabbit eyes and cornea. In situ gel was prepared in accordance with our previous reported method (27). Composition of developed in situ gel is given in Table VII. Conducted studies on developed formulation show a higher permeation across rabbit cornea after 4 h (64.21%) as compared to marketed formulation (47.11%) (Figure 5). This reveals the well-known bioadhesive and permeation enhancer property of chitosan. Generally, very small quantities of drug are able to permeate through cornea and reach the anterior chamber of eye for action. It is very difficult to quantify such small amounts of drug accurately. Researchers used cumbersome techniques like LC with mass spectrometry with hectic sample preparation for its evaluation. UPLC can analyze accurately the minute quantity of drugs, even in the nanogram range. Here we tested newly launched UPLC technique and found it satisfactory for quantification of such minute quantity of drug in aqueous humour and its application in ocular pharmacokinetic study.

Estimation of formulations

The applicability of the validated method was also tested by analyzing levofloxacin in pharmaceutical dosage forms, such as tablets and eye drops. The assay values of levofloxacin for different formulations represent the average of six individual assays. Levofloxacin tablet, which was claimed to contain 500 mg of levofloxacin, gave a mean assay value of 501.97 ± 1.3946 mg. Recovery and %RSD of the assay method were 100.4 and 0.28%, respectively, whereas the levofloxacin eyedrop that was claimed to contain 0.5% (w/v) of levofloxacin, gave a mean assay value of 5.02 ± 0.0695 mg/mL with a recovery and %RSD of 100.4 and 1.37, respectively. Assay values of formulations were found to be close to the claimed value, indicating that the interference of excipient matrix is insignificant in estimation of levofloxacin by the proposed UPLC method. The estimated drug content with low values of RSD (< 2%) established the precision of the proposed methods. The results obtained are compiled in Table VIII.

Conclusion

The newly developed reversed-phase UPLC method was found to be suitable for levofloxacin determination in aqueous humour, bulk drug, and pharmaceutical formulations

with fast retention time and maintaining good resolution. The method was completely validated, showing satisfactory data for all the parameters tested. It is also a stability-indicating method, which exhibits an excellent performance in terms of sensitivity and speed. Moreover, because the method does not imply any sample preparation step, only a small volume of aqueous humour sample is required, and a rapid determination of levofloxacin can be performed.

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