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ORIGINAL ARTICLE

RP-HPLC-UV validation method for levofloxacin hemihydrate estimation in the nano polymeric ocular preparation

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Abstract The specific and accurate reversed-phase HPLC-UV method has been validated to determine levofloxacin hemihydrate (LEVH) level. The separation was conducted at C 18 analytical column by administering mobile phase acetonitrile, methanol, and phosphate buffer (pH 3) with the ratio of 17:3:80. The flow rate of the mobile phase was 1 mL/min with a UV detector at 295 nm wavelength. Analytical methods validation evaluated includes specificity, linearity, accuracy, precision, LOD, LOQ, and robustness. The implementation of the analytical method was employed to determine LEVH level in ocular polymeric nanoparticles preparations. The test was specific for LEVH with the retention time of 7.66 min. Linearity was obtained from the concentration range of 4.8 μg/mL to 29.04 μg/mL. All method validation criteria are within the acceptable range. The developed method can be applied for LEVH polymeric nano-formulation analysis.

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1. Introduction

Bacterial keratitis is an infectious disease causing vision loss or blindness (AlMahmoud et al., 2019; Farahani et al., 2017). Based on the microorganisms' culture and sensitivity test, the disease can be overcome by prescribing proper antibiotics. Levofloxacin (Fig. 1), a third-generation fluoroquinolone antibiotics, can treat bacterial keratitis by inhibiting DNA-gyrase and topoisomerase IV (Blondeau, 2004; Kasetsuwan et al., 2011; Kowalski et al., 2010; Wong et al., 2012). Levofloxacin is available in 5 mg/mL eyedrops solution, however, due to the anatomic and physiological constrictions of the eye, topical administration of the drops results on low bioavailability (Addo et al., 2016; Ameeduzzafar et al., 2018; Cholkar et al., 2013; Kaskoos, 2014; Patel et al., 2012). This bioavailability issue can be addressed by applying nanoparticles to increase permeability across biological membranes, increase drug availability in the cul-de-sac and extend residence time, and sustain the release of the drug (Ghafoorianfar et al., 2020; Weng et al., 2017).

The entrapment efficiency percent of nanoparticles (% E.E.) is an essential parameter to be determined in formulation. Specific and accurate measurement of the value of % E.E. is essential to ensure just right amount of levofloxacin to be delivered by the preparation. Some options have been introduced to determine levofloxacin in the preparations and biological fluids matrix, including HPLC-UV (Dafale et al., 2015; Helmy, 2013), HPLC LC-MS (Fang et al., 2010; Xu et al., 2011), HPLC-PDA (Locatelli et al., 2015), U.V. spectroscopy, HPLC-fluorescent spectroscopy, 1H NMR spectroscopy, UPLC Vis spectroscopy, and capillary electrophoresis (Czyrski, 2017).

When HPLC system is opted, mobile phase to be employed for levofloxacin analysis is a combination of water or aqueous buffers and organic solvents. Triethanolamine (TEA) may be added in the mobile phase to improve peak shape (Czyrski and Szałek, 2016; Locatelli et al., 2015; Watabe et al., 2010); Other than that, sodium or potassium phosphate buffer in the 10-30 mM concentration range can be used (Helmy, 2013; Locatelli et al., 2015). Acetonitrile in the range of 14–43% in isocratic elution is the most frequently used organic solvent for HPLC separation (Czyrski and Szałek, 2016; Locatelli et al., 2015; Sousa et al., 2012; Xu et al., 2011).

The determination of % E.E. of nanoparticles for levofloxacin has been performed using a U.V. spectrophotometer (Gevariya et al., 2011; Gupta et al., 2011; López-López et al., 2019, p.). HPLC-UV with acetic acid and acetonitrile as mobile phases (Ameeduzzafar et al., 2020), and HPLC-UV with H_20 (pH3) and methanol as mobile phase (Siafaka et al., 2019).

This study validated the analytical method to determine levofloxacin hemihydrate (LEVH) concentration using HPLC-UV with acetonitrile, methanol, and phosphate buffer (pH 3) as mobile phase. The method was then applied to measure % E.E. of LEVH ocular nanoparticles. This study reported selectivity, linearity, accuracy, precision, LODs, LOQ, and robustness as guided by the International Harmonization Conference (ICH).

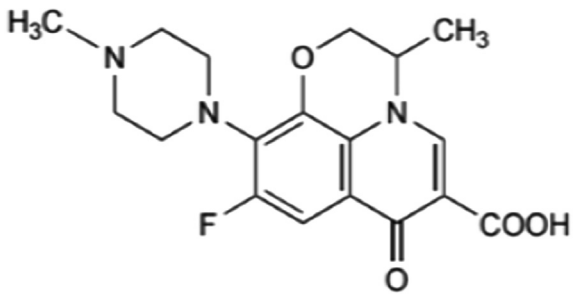

Fig. 1 Chemical structure of levofloxacin.

2. Experimental procedure

2.1. Instrumentation

A Hitachi L-2000 HPLC with an L2130 pump and an L-2420 UV–Vis detector was used for chromatographic separation. A manual injector system with a 20 µL injector loop and a 50 µL syringe (Hamilton, Microliter 705LT) was applied. A Luna Phenomenex® C18 (250 × 4.6 mm; 5 µm) was used as column, and the D-2000 Elite software controlled the HPLC apparatus and data collecting. Analytical balance, *vortex mixer* (Maxi-MixTM), pH meter (Hanna), a 0.45-µm PTFE membrane filter (Phenomenex®), nylon syringe filter 0.22 µm, glassware (Pyrex), micropipette (Biologix, USA), and ultrasonic bath were used for sample preparations.

2.2. Materials

Analytical grades of levofloxacin hemihydrate (LEVH) and ciprofloxacin (CPR) (Sigma-Aldrich, Buchs, Switzerland) were obtained from P.T. Pharos Indonesia Tbk (Indonesia). Other materials used in this study were potassium dihydrogen phosphate, sodium hydroxide, orthophosphoric acid, acetic acid, hydrochloric acid analytical grade, acetonitrile (ACN), and methanol (MeOH) HPLC grade (Merck, Darmstadt, Germany), Aquabidest (Ikapharmindo, Jakarta, Indonesia) and purified water (Onemed, Surabaya, Indonesia). As excipient materials of the nanoparticles, chitosan medium molecular weight and pectin (*Sigma-Aldrich*, Buchs, Switzerland) were used.

2.3. Buffer preparation

Three grams of potassium dihydrogen phosphate was dissolved with distilled water in a 1000 mL volumetric flask. The pH was then adjusted with 180 µL of phosphoric acid to pH 3.0, and volume was adjusted to 1 L with water. The solution was filtered using a 0.45 µm membrane filter (Phenomenex®) and ultrasonic-degassed for 15 min.

3. Methods

3.1. Chromatographic conditions

Isocratic elution was implemented in the study. Acetonitrile, methanol, and phosphate buffer pH 3.0 (17:3:80 v/v/v) were used as mobile phase, flowing at 1 mL/min through a Luna Phenomenex® C18 (250 4.6 mm; 5 m) column. Per run, a volume of 20 µL of the sample was injected and disclosed with a U.V. detector set at 295 nm.

3.2. Preparation of standard solution and calibration curve

As internal standard, 10 mg of LEVH and CPR were placed into a 10.0 mL volumetric flask, dissolved with sufficient acetic acid, and diluted with mobile phase to attain a concentration of 1 mg/mL. The standard solution was diluted with the mobile phase to obtain LEVH concentration of 4.84; 9.68; 14.52; 19.36; 24.20; and 29.04 µg/mL. Each concentration of standard LEVH solution contains an internal standard as

Table 1 Result of the suitability test of levofloxacin and ciprofloxacin HPLC systems in the mobile phase ACN:MeOH: phosphate buffer pH 3 (17:3:80).

Parameters	LEVH (n = 6)		CPR (n = 6)	
	Average	RSD (%)	Average	RSD (%)
Retention Time (minutes)	7.66 ± 0.01	0.11	8.44 ± 0.01	0.15
Area	563189.33 ± 6301.42	0,81	879621.00 ± 9945.98	0.69
Tailling Factor	1.18 ± 0.01	1.02	1.21 ± 0.02	1.22
Resolution	2.51 ± 0.02	0.5	2.51 ± 0.02	0.5
Number of Theoretical Plates	10748.17 ± 166.83	1.19	10963.50 ± 160.81	1.23

much as 20 μg/mL. Calibration was done by curve-fitting concentration to LEVH to CPR area ratio.

3.3. Analysis method validation

3.3.1. System suitability test

System suitability test was conducted by injecting six replications of the standard solution before the analysis. The observed parameters were relative standard deviation (% RSD) for peak area and retention time, tailing factor, resolution, and number of theoretical plates.

3.3.2. Specificity

Method's specificity was tested by comparing levofloxacin chromatogram and ciprofloxacin with the chromatogram of the nanoparticles matrix. The matrix was used to observe the effect of excipients on the retention time of levofloxacin and ciprofloxacin analytes.

3.3.3. Linearity

Linearity test was conducted by assessing the ratio of each area of 4.84; 9.68; 14.52; 19.36; 24.20; and 29.04 μg/mL LEVH and 20 μg/mL CPR to the common area.

3.3.4. Accuracy

Determination of the recovery value in this study was performed at three different concentrations: 4.8 μg/mL, 14.5 μg/mL, and 29.04 μg/mL.

3.3.5. Precision

Precision is determined by the fulfilment of coefficient of variation (RSD) value to 2% of intra- and inter-day examinations.

3.3.6. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated by the signal-to-noise ratio of three and ten times respectively.

3.3.7. Robustness

Rigidity test was performed by diversifying the maximal wavelength (294 and 295 nm) and the speed of flow time (0.9 and 1 mL/min).

3.4. Nanoparticles preparation

0.1% w/v Levofloxacin nanoparticle containing chitosan (0.1% w/v) and pectin (0.02 w/v) was prepared by mixing 500 μL of chitosan solution and 500 μL of LEVH into a microtube, homogenized using a vortex mixer for 60 s at 1250 rpm. Then, 500 μL of pectin solution was added and vortex-mixing was continued for 60 s.

3.5. %E.E. Determination

%E.E. of LEVH in nano polymeric formulation was determined by indirect method. The dispersion were centrifuged for 50 min at a speed of 15000 rpm. Twenty microliters of supernatant were taken, CPR standard solution was added and then diluted using mobile phase ad 1 mL. A total of 20 μL of sample were injected into the column, and then % E.E. was calculated using Eq. (1).

$$\%EE = \frac{\text{The amount of the drug} - \text{the amount of the drug in the supernatant}}{\text{the amount of the drug}} \times 100\% \quad (1)$$

4. Results

4.1. System suitability test

Measured parameters fulfilled the criteria of acceptance. The RSD of retention time and area < 2%, tailing factor < 2, N (number of theoretical plates) > 2000, and Rs (resolution) > 2 as presented in Table 1.

4.2. Specificity

LEVH peaks (Rt = 7.66 min) and ciprofloxacin peaks (Rt = 8.43 min) were well separated with a value of Rs > 2. The results of the nanoparticle matrix chromatogram presented that no peaks occurred at 7.66 and 8.43 min (Fig. 2.). The result indicates that the HPLC assay possesses good selectivity for levofloxacin assay administering the standard internal method.

4.3. Linearity

The obtained linear regression equation is Y = 0.1097x + 0. 0439, with the correlation coefficient of 0.9998 (Table 2), indicating qualified linearity parameter. The levofloxacin standard curve is displayed in Fig. 3.

4.4. Accuracy

The value of % recovery generated as shown in Table 3 fulfills the requirements of % recovery in the range of 80–110%.

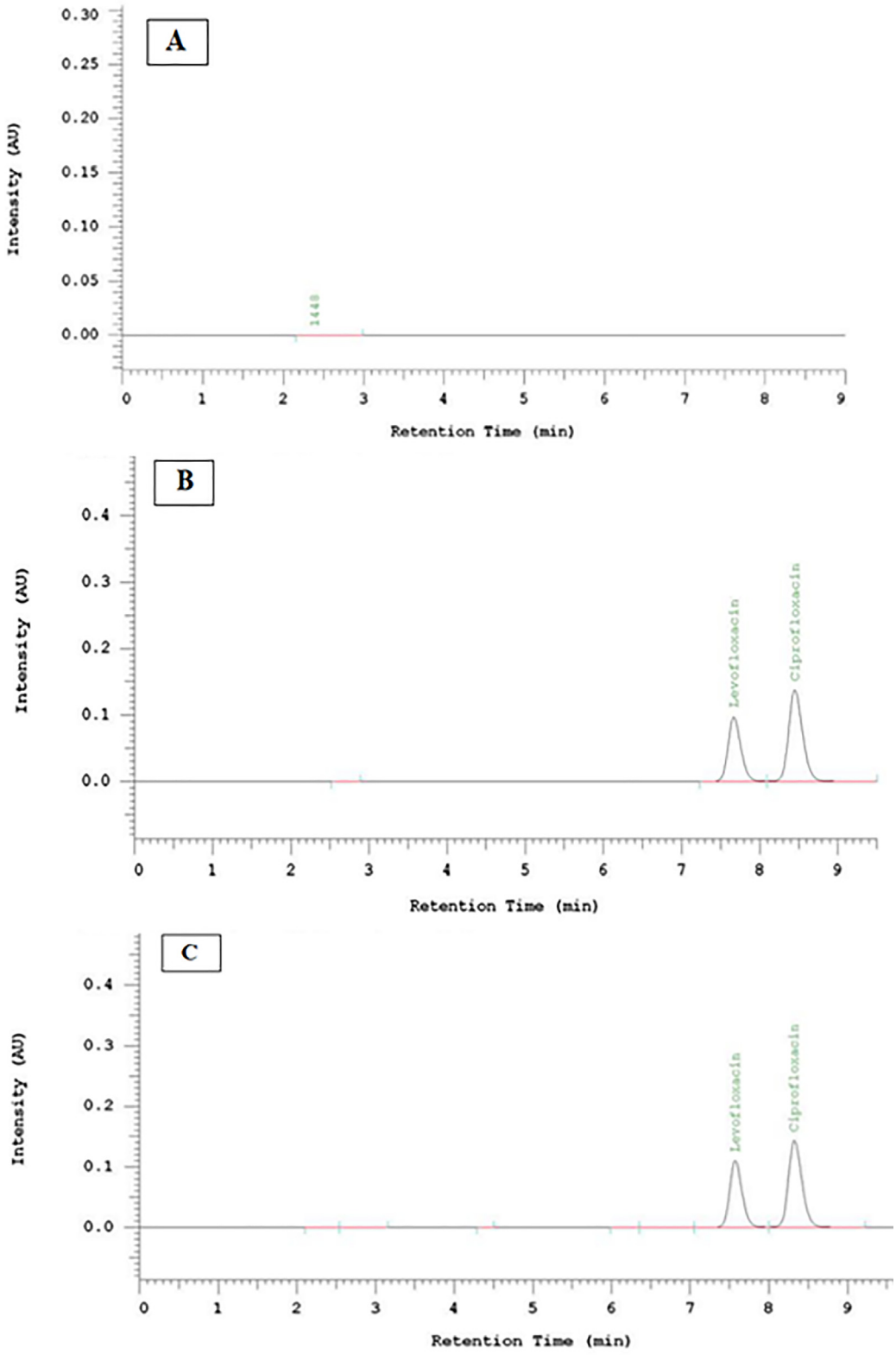

Fig. 2 Chromatograms of nanoparticle matrix (A), levofloxacin and ciprofloxacin (B), and levofloxacin-unloaded nanoparticle (C).

Table 2 Regression characteristics of levofloxacin hemihydrate calibration curve.

LEVH concentration (μg/mL	Area Ratio
4.84	0.578
9.68	1.126
14.52	1.625
19.36	2.150
24.20	2.676
29.04	3.261
Correlation Coefficient (r)	0.9998
Slope	0.1097
intercept	0.0439

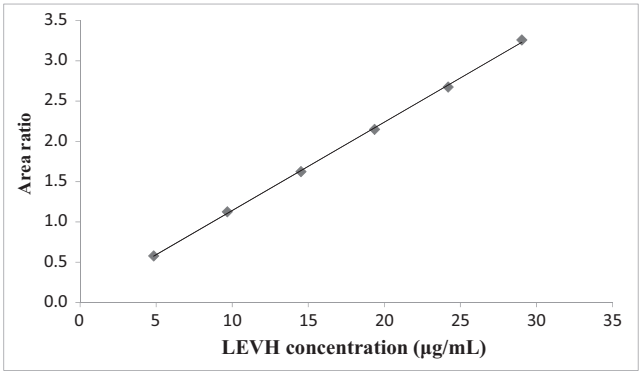

Fig. 3 Standard curve of Levofloxacin.

Table 3 Recovery study of LEVH.

Theoretical concentration (μg/mL)	Measured concentration (μg/mL ± SD) (n = 3)	% Recovery
4.8	4.88 ± 0.02	100.92 ± 0.33
14.5	14.53 ± 0.01	100.05 ± 0.70
29.04	29.35 ± 0.04	101.09 ± 0.12

4.5. Precision

The results of the intermediate precision intra- and inter-day testings showed the required precision (RSD < 2) (Table 4).

4.6. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ values obtained in the method analysis were 0.66686 μg/mL and 2.22286 μg/mL, respectively, based on the standard curve equation.

4.7. Robustness

The results showed that the RSD value is <2 (Table 5), indicating fulfilment of robustness criteria.

Table 4 Intermediate precision study of LEVH.

Day	Actual LEVH concentration (μg/mL)	Measured concentration (μg/mL) (n = 3)	%R.S.D
1	4.8	4.88 ± 0.02	0.32
	14.5	14.53 ± 0.08	0.70
	29.04	29.88 ± 0.15	0.51
2	4.8	4.86 ± 0.04	0.75
	14.5	14.42 ± 0.03	0.22
	29.04	29.35 ± 0.04	0.12
3	4.8	4.98 ± 0.01	0.11
	14.5	14.36 ± 0.08	0.54
	29.04	29.56 ± 0.15	0.50

5. Discussion

5.1. Chromatographic conditions

Reverse-phase HPLC method is used to separate LEVH. Commonly used in reverse phase HPLC is C18 bonded to the silica surface as the stationary phase due to the chemical interaction between chlorosilane and silanol groups. LEVH and CPR are polar, therefore the elution power of the mobile phase must be decreased to increase interaction between the analyte and the stationary phase. Combining solvents ACN (proton acceptor) with methanol (proton donors) increases selectivity. Interactions between the analyte and stationary phase are generally hydrophobic, and secondary interactions with silanol residues can cause tailings in compounds with amine groups so that pH adjustment is carried out to prevent the ionization of silanol residue. LEVH is base for its amine group on the piperazine ring. The interaction between positively ionized amine base groups ($-NH_2^+-$) and negatively ionized silanol residues (SiO–) causes peak tailing thus reduces resolution. This adverse secondary interaction can be avoided by keeping the silanol residue in the unionized form. At pH > 8, the silica as a solid support of the stationary phase can be dissolved, while at pH < 2, siloxane linkages can be hydrolyzed in the stationary phase. Consequently, a generally safe and effective mobile phase may be performed at pH 3 with a suitable buffer, one of which is a phosphate buffer (a combination of H_3PO_4 and KH_2PO_4) (Nemutlu et al., 2007; Sousa et al., 2012).

UV spectrophotometer detector was used. The levofloxacin compound has chromophore and auxochrome groups to be detected with this detector. Ciprofloxacin was used as an internal standard because it has physical and chemical properties similar to LEVH. Internal standard procedure was used to precisely and sensitively assess levofloxacin level. The developed method can provide good separation with < 10 min of retention time so that analytical methods may be used to determine levofloxacin level in pharmaceutical formulations.

5.2. Implementation of the analytical method in polymeric nanoparticle formulas

The % E.E.value id the quantification of the amount of drug absorbed in the nanoparticle preparation. The test was carried out by indirect method. The % E.E. is 21.34 ± 1.53% with unloaded nanoparticles and LEVH chromatograms showed

Table 5 RSD value in the levofloxacin robustness test with a concentration of 4.8 μg/mL.

Variation condition	Flow rate 0.9 mL/min			Flow rate 1.0 mL/min		
	Measured concentration	% recovery	R.S.D	Measured concentration	% recovery	R.S.D
Wavelength 294 nm	4.8	4.88 ± 0.02	0.32	4.8	4.88 ± 0.02	0.32
Wavelength 295 nm	14.5	14.53 ± 0.08	0.70	14.5	14.53 ± 0.08	0.70

similar retention time. Furthermore, no peaks presented at the retention time of LEVH and CPR in the chromatogram of the nanoparticle matrix. This result indicates that the developed method could specificity determine LEVH levels in polymeric nanoparticles preparations.

6. Conclusions

The development and validation methods of HPLC for analyzing LEVH using acetonitrile: methanol: phosphate buffer pH 3 (17:3:80) is specific, accurate, precise, and robust. The system can be implemented to analyze LEVH in polymeric nanoparticles.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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