A NOVEL VALIDATION OF THE LEVOFLOXACIN DETERMINATION METHOD IN URINE (IN VITRO) USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-UV/VIS DETECTOR

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ABSTRACT

Levofloxacin (LFX) is a fluoroquinolone antibiotic, which so far, has no method of determining its level in urine using solid-phase extraction (SPE) technique followed by quantification with high-performance liquid chromatography (HPLC) with a UV-Vis detector. The purpose of this study is to find a selective and fast method for examining levofloxacin in the urine. This analysis used a high-performance liquid chromatography method, using a C18 inverse phase column, the stationary phase (column) used was octadecyl silane (ODS) 250 x 2.6 mm, 10 μ m particle size, using a mixture of phosphate buffer: acetonitrile (85.5: 14.5, v/v) pH 2.5 as a mobile phase, with a flow rate of 1.2 mL/min and using ciprofloxacin (CFX) as internal standard (IS). Levofloxacin was detected at a wavelength of 295 nm using a UV detector with a levofloxacin retention time of 9–10 minutes and a resolution of 1.7 from urine. Linearity was obtained by using the internal standard of ciprofloxacin 10 μ g/mL with a concentration of levofloxacin 1.15, 2.8, 5.75, 11.5, and 23 μ g/mL, the correlation coefficient (r) = 0.999. The limit of detection (LOD) of levofloxacin and limit of quantitation (LOQ) and were 0.0733 μ g/mL and 0.0220, respectively. The percent recovery based on an area of 1.15 and 23 μ g/mL was 106.84% and 89.37%, based on the peak height of 109.52 μ g/mL and 91.90 μ g/mL, relative standard deviation (RSD) precision (%) respectively 1.09% and 0.45% based on area and based on peak heights of 12.66% and 1.13%. The concentration accuracy value of 1.15, 5.75, and 23 μ g/mL were 86.23%, 99.98%, and 97.95% while the precision was 2.78%, 5.27%, and 1.14%. The results of the system suitability test and repeatability to retention time, area ratio, and peak height showed coefficient variation RSD <15%. The results of the validation method meet the requirements so that it can be used to determine levofloxacin in the urine for routine analysis

Keywords: Validation, Levofloxacin, Urine, HPLC, UV-Vis.

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INTRODUCTION

Levofloxacin (LFX) is a broad-spectrum antibiotic that is effective against many Gram-positive and Gram-negative bacteria, so levofloxacin is often used empirically for widespread infections (pneumonia, urinary tract infections before specific causes are known).^{1,2} Levofloxacin is a relatively new fluoroquinolone antibiotic and it is a drug of choice for infections. However, the method of determining the levels is not widely known, especially in biological fluids such as from blood and urine, so that research is needed to get a simple, fast, accurate, and validated analytical method for pharmacokinetic and pharmacodynamic analysis purposes.^{3,4}

Validation of the analytical method is an action taken as proof of certain parameters that are required and determined so that the analysis carried out gets the desired results. This validation is done because of differences in tools, limitations of equipment, chemicals or other conditions that ensure the method cannot

be applied as a whole, so it is necessary to modify, simplify and improve the method by using solid-phase extraction (SPE) by HPLC with a UV detector.⁵ This analysis method requires several parameters, which include linearity, precision, accuracy, detection limit (LOD), and quantitation limit (LOQ).^{6,7}

The development of levofloxacin determination in pharmaceutical preparations has been carried out by conductometry, potentiometry, spectrophotometry,⁸ and by HPLC with UV detectors⁹, while in biological fluids (plasma/urine) HPLC has used fluorescence detectors.^{3,5,10,11} diode array detectors, and spectrometers mass.¹² Several procedures and techniques have been developed to determine levofloxacin levels in the biological liquid matrix, including the HPLC method^{1,13,14}, microdialysis,³ fluorimetry,^{15,16} and chemiluminescence,⁸ anodic stripping voltammetry,¹⁷ potentiometry,⁸ flow injection spectrophotometric¹⁸, and LC-MS.¹⁹ Based on research that has been carried out, there is no combination of levofloxacin analysis using HPLC-SPE-UV-Vis detector. This research is intended to produce a simple, fast, and accurate method that can be used for levofloxacin analysis in biological fluids, which is for routine analysis purposes.

EXPERIMENTAL

Materials

The material used in this analysis was Levofloxacin Comparative Raw (Zhejiang Jinxin-China), ciprofloxacin (Zhejiang, Jinxin, China). Sodium dihydrogen phosphate dihydrate p.a (Merck®, Germany), acetonitrile p.a (Merck®, Germany), and phosphate acid (Merck®), methanol pro liquid chromatography (J.T Baker, USA), and aquabidest (IPHA Pharmindo, Indonesia).

Tools

The equipment used in this analysis is a set of high-performance liquid chromatography tools (Shimadzu LC-10 ATVP, Japan) which consists of an SPD UV-VIS detector, Shimadzu SCL-A System Auto Controller injector, HPLC column (Phenomenex, Germany), 250 mm long, inner diameter of 2.6 mm, particle size of 10 µm, a set of UV-Vis spectrophotometer (analytical Jena, specord 200, Germany), pH meter (Ohmeter), Germany, ultrasonic bath (NEY 1510), SPE cartridge HLB 30 mg 1 cc (Oasis, Swiss).

Determination of A UV-Vis Wavelength for Levoflocin

Preparation of Mobile Phase

Sodium dihydrogen phosphate dihydrate weighed (1.7248 g), was dissolved into a 500 mL volumetric flask, diluted with aquabidest to the limit mark, Then added a drop of phosphate acid was added dropwise to obtain a phosphate buffer with a pH of 2.5 using a pH meter. The mobile phase used is the ratio between the phosphate buffer 0.025 M pH 2.5 with acetonitrile (85.5:14.5 v/v).

Preparation of Levofloxacin Calibration Curve

Standard of a levofloxacin was accurately weighed as much as 50 mg, dissolved in a 100 mL volumetric flask using the mobile phase to the boundary markers so that the final concentration of 0.5 mg/mL was obtained.

Determination of Maximum Wavelength of Levofloxacin

The final concentration of the standard solution is 5×10^{-3} mg/mL, then the solution is cleansed with an ultraviolet spectrophotometer at a wavelength of 200–380 nm, to obtain the maximum wavelength of levofloxacin.

Calculation of Molar Extinction (ε)

Levofloxacin standard solution of 0.5 mg/mL was prepared series of solution (6.4, 10.8, and 13.5 µM) using 0.05 M HCl in a 50 mL volumetric flask. The three solutions were then analyzed by spectrophotometer, and the absorption at a maximum wavelength of 295 nm was determined, and the molar extinction values were determined using the following expression:

$$\epsilon = A / b C$$

b = thick of cuvet (cm) ; C = consentration (Molar)

Determination of Optimum HPLC Condition

To obtain the optimum conditions of levofloxacin with SI ciprofloxacin, experiments with various combinations of mobile phases were appropriate (Table-1).

Table 1. Optimation for a HPLC analysis condition.

Column	Octadecyl silane/C ₁₈ (Phenomenex), length 250 mm, internal diameter dalam 2.6 mm, and particle size 10 µm
Mobile phase	Sodium dihydrogen phosphate (0,025 M, pH2,5) : acetonitrile (85:15 v/v) and (85,5: 14,5 v/v)
Flow rate	1.0 dan 1.2 mL/minute
Injection volume	10 µL
Internal standard	10µg/mL ciprofloxacin
Detection	UV 295 nm

Extraction Using SPE (Oasis HLB 1CC)

SPE cartridge conditioning was conducted using methanol:water (1:3 mL), loading sample stage was employed by put on the sample concentration to be measured, then carried out washing stage using 3% methanol in aquabidest, and the last stage was eluting, using 20% ACN in a phosphate buffer 0.025 M at pH 2.5. The results of elution were collected for HPLC analysis.

Recovery Extraction for SPE

Recovery extraction was carried out to concentrations 1.15 and 23 µg/mL, and it was calculated using equation (1) below:

$$\% \text{ Recovery} = \frac{\text{Peak Area Plasma Chromatogram}}{\text{Peak Area Mobile Phase Chromatogram}} \times 100\% \quad (1)$$

HPLC Validation Method for Determining Level of Levofloxacin

Determination of Selectivity

Selectivity was expressed by the value of resolution or separability (R_s) ≥ 1.5 . It was calculated using equation (2):

$$R_s = [2 (t_2 - t_1) / (W_1 + W_2)] \quad (2)$$

Where, T = retention time and W = wide at 10% of peak).

Repeatability Test

At the optimum condition, the levofloxacin standard solution concentration of 1.15 µg/mL in urine, as much as 10 µl was injected into the HPLC, the experiment was repeated six times then the coefficient of variation was calculated and stated as RSD (%).

Linearity Studies

Five series of levofloxacin were made with concentrations (1.15, 2.8, 5.75, 11.5, and 23 µg/mL) using the internal standard of ciprofloxacin with a concentration of 10 µg/mL in urine, then extracted using SPE with the conditioning step. Linear equations were determined to determine the correlation coefficient (r) between the concentrations of levofloxacin from the ratio of the area of levofloxacin to the internal standard of ciprofloxacin, with a correlation coefficient value >0.99 .

Determination of LOD and LOQ

The detection limit and the quantitation limit for levofloxacin content determination can be calculated statistically from a calibration curve equation using a linear regression line.

The limit of detection (LOD) is stated with equation (3):

$$\text{LOD} = \frac{3S_b}{a} \quad (3)$$

The limit of quantitation (LOQ) was calculated using equation (4):

$$LOQ = \frac{10S_b}{a} \quad (4)$$

Test of Accuracy and Precision

Accuracy and precision on the same day were obtained by determining the levels of three samples ($n = 3$) for three times in a row. The accuracy was obtained by looking at the closeness of the results of the sample to the nominal value and precision seen from the RSD (%), recovery is calculated by equation (5):

$$\% \text{ Recovery} = \left[\frac{CT}{CA} \right] \times 100\% \quad (5)$$

Where, CT is the measured concentration of levofloxacin, and CA is the amount of levofloxacin that is administered (nominal concentration) into the urine. Precision is expressed as RSD (%), by equation (6):

$$\%RSD = \left(\frac{SD}{\bar{X}} \right) \times 100\% \quad (6)$$

System Suitability Studies

From the chromatogram obtained, for each concentration determined, repeat the injection of a standard solution expressed in RSD (%) less than 15% of the retention time, area ratio, and peak height ratio of the chromatogram. The system suitability test was carried out on levofloxacin samples with a final concentration of 1.15 (low concentration) and 23 $\mu\text{g/mL}$ for (high concentration) in urine, with an internal standard of ciprofloxacin, 10 $\mu\text{g/mL}$, respectively. Each repetition was carried out six times ($n = 6$) with an injection volume of 10 μL into the HPLC device at optimum conditions.

RESULTS AND DISCUSSION

Determination of Measurement Wavelength

The results of the wavelength of the standard solution of levofloxacin and ciprofloxacin as an internal standard, using a comparison of the mobile phase phosphate: acetonitrile (85.5:14.5) obtained the maximum wavelength of levofloxacin 295 nm and the maximum wavelength of ciprofloxacin 279 nm. The results of the analysis can be seen in Fig.-1. Ciprofloxacin is used as an internal standard due to it has the same structure with levofloxacin so that it can be seen the method to separate the two compounds.

Fig.-1: The Spectrum of Maximum Wavelength

Results of Molar Extinction

Levofloxacin can be detected using an ultraviolet-visible detector in the HPLC system because the average value of the levofloxacin molar extension (ϵ) is $33.002 \text{ M}^{-1}\text{cm}^{-1}$ (see Table-2) greater than the accepted value for molar extinction that is $10.000 \text{ M}^{-1}\text{cm}^{-1}$.²⁰ Its values showed a ability of Uv-Vis detection can be used for levofloxacin and ciprofloxacin.

Table-2: Molar Extinction of Levofloxacin HCl 0.1 N at wavelength 295 nm ($n = 3$)

n	Concentration (M)	Absorbance	Molar Extinction (ϵ) ($M^{-1}cm^{-1}$)
1	0.0000135	0.3896	28859.26
2	0.0000108	0.3624	33555.56
3	0.00000648	0.2343	36593.75
$\bar{X}$			33002.86

Determination of The Analysis Conditions Result

The results of determining the optimal conditions based on the conditions that produced resolution values of CFX and LPX peaks (RS) of more than 1.5 and can be seen in Table-3. Selection of the optimal analytical condition is based on the required separation and the fastest retention time of the analyte.

Table-3: Optimization Results of HPLC Conditions

Column	Octadecyl silane/C ₁₈ (Phenomenex), length 250 mm, internal diameter 2.6 mm, and particle size 10 μ m
Mobile Phase	Sodium dihydrogen phosphate (0,025 M, pH2,5) : acetonitrile (85.5: 14.5 v/v)
Flow rate	1.2 mL/minute
Volume injection	10 μ L
Internal standard	10 μ g/mL ciprofloxacin
Detection	UV 295 nm

ODS=Octadecylsilane

Recovery Extraction Value

The results of recovery extraction have shown in Table-4. were obtained based on the requirements to enter the range of 80–120% recovery, and the results of the coefficient of variation (RSD %) into the range for biological fluids are <15%.^{7,21,22} The extraction recovery value shows a good extraction method so that the sample can be drawn completely from the blood plasma.

Table-4: Recovery of Extraction Value ($n = 3$).

Peak Area			Height Peak		
	Concentration (μ g/mL)			Concentration (μ g/mL)	
	1.15	23		1.15	23
	93.43	88.92		93.57	90.72
n = 3	111.79	89.68	n = 3	111.27	92.40
	115.30	89.51		118.73	92.35
$\bar{X}$ (%)	106.84	89.37	$\bar{X}$	109.52	91.90
RSD %	10.9	0.45	RSD %	12.66	1.13

Notice: RSD= Relative Standard Deviation

Validation Method for Determining Levels of Levofloxacin by HPLC**Selectivity**

The results of selectivity can be seen in Fig.-2. The selectivity of a method can be seen from the value of separation between internal standard ciprofloxacin and levofloxacin, as samples showed a good resolution value. It means the method can separate LFX and CPX with a resolution value of ≥ 1.5 .²⁰

The Repeatability Test Result

The repeatability test was carried out using a levofloxacin sample concentration of 1.15 μ g/mL with an internal standard concentration of 10 μ g / mL, repeated with six replications. This test is said to meet the requirements if the value of the area retention time was based on RSD biological fluid <15%.²⁰ The analysis results obtained by area are 1.3% while based on 0.66% retention time so that the repeatability test results are said to meet requirements based on the validation parameters.

Fig.-2: Chromatogram of Separation of CFX and LFX (Selectivity).

Fig.-3: Chromatogram of Repeatability

Linearity Determination Result

The linearity test results presented in Fig.-4 and the results of the linear regression equation obtained are $(r) = 0.999$, the equation $Y = 0.143x + 0.121$. These results meet the linearity test requirements on biological fluids with $(r) > 0.995$; the value shows a comparable value between the increase in concentration and the response of the analysis (peak and height area).^{20,21}

Fig.-4: Chromatogram of Linearity

LOD and LOQ Values Result

The best correlation coefficient (r) results obtained from the levofloxacin standard curve can be used for testing the limit of detection (LOD). The LOD value of the area ratio is $0.0220 \mu\text{g/mL}$, and the LOD value based on peak height is $0.1679 \mu\text{g/mL}$. The LOQ test results were obtained based on the levofloxacin calibration curve of the equation that had the best correlation coefficient of the calibration curve. The LOQ results are obtained from the chromatogram area ratio calibration curve. The LOQ value of the chromatogram area ratio is $0.0733 \mu\text{g/mL}$, and the LOQ value at peak height was $0.5598 \mu\text{g/mL}$. Both LOD and LOQ values obtained can be used for both quantitative and qualitative analyses, especially in human urine. Values of LOD and LOQ can be concluded to have the ability to detect an LFX and CFX level in human plasma.

Accuracy and Precision Value

Determination of the precision and accuracy, three series of levofloxacin sample concentrations were made in the urine matrix. Low, medium, and high-value calibration curves were taken, respectively (1.15, 5.75, and 23 µg/mL), and contained an internal standard of ciprofloxacin 10 µg/mL, as shown in Table 4. The value of accuracy from the three concentrations in the range 86%–99% and the precise value at 1%–5%, were both accurate and precise and fulfilled the value as required (Table-5).^{20,22}

Table-5: % Accuracy and Precision value ($n = 3$)

Nominal Concentration (µg/mL)			Accuracy (% Recovery)			Precision (% RSD)		
1.15	5.75	23	1.15	5.75	23	1.15	5.75	23
0.96	5.42	22.33	86.23±2.40	99.98±5.27	97.95±1.12	2.79	5.27	1.14
0.99	5.80	22.44						
1.02	6.02	22.82						

The Experiment Conducted ($n = 3$) in intra day ($r = 1$), RSD = Relative Standard Deviation.

Suitability System Test Result

System suitability test results obtained from the concentration of 1.15 µg/mL, meet the requirements of the coefficient of variation $\leq 5\%$,^{20,23} as shown in Table-6.

Table-6: Values of Suitability System

Parameter	RSD (%)	
	Concentration of LFX (1.15 µg/mL)	
Retention time	LFX	0.66
	CPX	0.68
	Ratio	0.07
Peak area	LFX	1.30
	CPX	0.55
	Ratio	1.33
Height area	LFX	1.43
	CPX	0.36
	Ratio	1.32

CONCLUSION

Based on the results of the research, the following conclusions were obtained. Optimization of HPLC conditions and levofloxacin extraction with urine matrix, using SPE Oasis HLB 1cc, can be done with relatively good results. Based on the results of the validation of the analysis method with parameters of selectivity, accuracy, repeatability, linearity, the limit of detection, the limit of quantitation and suitability of the system, the results obtained showed that the method was carried out according to the required standards so that it can be used to determine levofloxacin using HPLC with a UV detector.

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