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OFLOXACIN ANALYSIS VALIDATION METHOD IN HUMAN BLOOD PLASMA (IN VITRO) USING SOLID-PHASE EXTRACTION HPLC

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Until now, analysis of Ofloxacin in human blood plasma using solid-phase extraction (SPE) by HPLC UV detector has not been reported. This study aims to determine the validity of analytical methods in Ofloxacin study in human blood plasma (in vitro) using an HPLC SPE UV detector. Plasma samples were extracted by SPE. Analytes were analyzed using a C18 column (octadecylsilane) 250x4.6 mm, particle size 10 μm, mobile phase 85,5:14,5 v v 0.025 M phosphate buffer (pH 2.2) and acetonitrile with a flow rate of 2 ml/min, detection performed at 294 nm with the internal standard ciprofloxacin. Validated analytical method was based on the parameters: selectivity, accuracy, precision, repeatability, linearity, LOD, LOQ, and the suitability of the system. Validation analysis showed selectivity test Rs>1.5, test repeatability with CV(%) <10%, linearity was obtained in the range of 0.1 to 6 μg/ml with correlation coefficient (r) from 0.9998 to 0.9999. Based on the area ratio of peak height and a segment of the chromatogram obtained LOD values 0.023 and 0.024 μg/ml, LOQ value of 0.076 and 0.080 tg/ml, percent accuracy from 94.32 to 100.45% and 97.68 to 101.63%, and precision CV (%) 0.31 to 0.85% and 0.84 to 1.08%. System suitability test results on the retention time, area ratio, and high ratios of peak chromatogram shows the CV(%) <10%. Can be concluded that the analytical methods used have validity in accordance with the requirements.

Keywords: HPLC, ofloxacin, SPE, human plasma.

UDC: 615.2

Introduction

Ofloxacin is an antibiotic second generation quinolones are broad spectrum. Ofloxacin is widely used for eye infections, urinary tract infections, gastrointestinal infections, respiratory infections, skin and soft tissue infections, joint and bone infections, pneumonia resistant to beta-lactam antibiotics and macrolide, and diseases that are transmitted through sexual contact. Ofloxacin working mechanism to inhibit bacterial protein synthesis inhibited the enzyme topoisomerase II (DNA gyrase) and IV (SG Ganiswarna et al., 1995; Nepali et al., 2007; Sultana et al., 2007; Tanwar et al., 2007).

A previous study reported that Ofloxacin analysis has been done in matrices of biological fluids such as protein precipitation using high performance liquid chromatography (HPLC) with UV detector (Rehak, 2004), with a fluorescence detector (Esposito, 2006; Wacke et al., 2006) and photodiode array detector (Baruah et al., 2004), solid-phase extraction method is HPLC with fluorescence detector (Rose et al., 1998) and photodiode array detector (Lai et al., 1997). Until now, analysis of research Ofloxacin in human blood plasma using solid-phase extraction (SPE) by HPLC UV detector has not been reported. SPE is an extraction method is more effective than other extraction methods, one can isolate the benefits of sample (analyte) is very small concentration in a matrix (Snyder et al., 1997).

Based on the structure of the chromophore Ofloxacin-containing clusters, the analysis of human blood plasma was isolated using SPE can be done by HPLC with UV detector,

with optimum results and has good validity. This research can be used as a reference in bioavailability and bioequivalence testing of Ofloxacin in the pharmaceutical industry or in clinical laboratories, particularly in Indonesia.

Materials and method

Material

Ofloxacin (Qualitative Analysis Chemistry Laboratory, Faculty of Pharmacy, Universitas Padjadjaran), ciprofloxacin (Jinxin Zhejiang, China). All chemicals were used as received without further purification and all solvents were of reagent grade: sodium dihydrogen phosphate monohydrate (Merck), acetonitrile, and phosphoric acid (Merck), methanol pa (Merck), aquabidest (IPHA), human blood plasma (Indonesian Red Cross, Bandung).

Tool

A set of tools HPLC (Shimadzu LC-10 ATVP) equipped with UV-VIS detector SPD, auto injector Shimadzu system controller SCL-A, the HPLC column (Phenomenex); length of 250 mm, 4.6 mm internal diameter, particle size 10 μm, a set of UV-Vis spectrophotometer (Analytical Jena, specord 200), pH meter (Ohmeter), ultrasonic bath (Ney 1510), HLB 30 mg SPE cartridge 1 cc (Oasis), an analytical balance (Sartorius) sensitivity of 0.1 mg, filters vacuum with 0.4 to 0.45 μm pore filter, and an unusual glass ware.

Method

Mobile phase was a mixture of 0.025 M phosphate buffer pH 2.2 and acetonitrile (85:15). The mixture was filtered using 0.45 μm milipore with vacuum assistance and ultrasonic bath for 15-20 minutes.

Standard Solution Preparation

Ofloxacin 100 mg dissolved in 200 ml measuring flask with mobile phase to achieve the final concentration of 0.5 mg/ml, diluted with mobile phase to obtain concentrations of 5 μg/ml. In-scanning solution with a UV-spectrophotometer at a wavelength of 200-320 nm, so the obtained spectrum maximum wavelength of absorption and ofloxacin. The same procedure done on ciprofloxacin.

Determination of molar extinction

Ofloxacin standard solution with a concentration of 6.9, 13.5, and 18.0 μM measured at a wavelength of maximum absorbance ofloxacin, and the calculated values molarnya extinction.

Optimization of HPLC conditions. Ofloxacin standard solution 0.1 mg/ml containing the internal standard ciprofloxacin 0.1 mg/ml was injected with 10 μl (auto injector) into the HPLC mobile phase composition of 85:15, 85,5:14,5, and 86: 14 v/v and flow rate was 1.2 and 1.3 ml/min. Viewed retention time and separation of the two peaks (ofloxacin and ciprofloxacin) were produced.

Extraction by SPE

Into the SPE cartridge added 1 ml of methanol and 1 ml aquabidest with vacuum assistance. Added 1 ml of plasma that had been in-spike with ofloxacin (0.10, 0.25, 1.00, 2.00, 3.00, 4.00, 5.00, and 6.00 μg/ml) and ciprofloxacin (3 μg/ml at each concentration ofloxacin) drop by drop. Added 1 ml 5% methanol. Analyte was eluted with 1ml acetonitrile 20% (in phosphate buffer). Analyte was collected and injected into the HPLC and SPE extraction efficiency was calculated.

Method validation analysis

Selectivity was determined by looking at the chromatogram ofloxacin and ciprofloxacin were the result of HPLC separation, calculated the value of the resolution. Repeatability was determined by making a solution of ofloxacin 0.25 μg/ml in blood plasma, and then extracted using SPE. 10 μl of analyte injected into the HPLC equipment in optimum

condition, the experiment was repeated six times and then calculated the coefficient of variation. The linearity was determined by making the standard curve of five serial concentrations of ofloxacin (0.10, 0.25, 2.00, 4.00, and 6.00 μg/ml) and the internal standard ciprofloxacin 3 μg/ml in blood plasma. Then extracted using SPE.

10 μl of analyte injected into the HPLC equipment in optimum condition. The experiment was repeated three times. Calibration curve equation with the best correlation coefficient was used to specify the sample. LOD and LOQ calculated statistically from the calibration curve equation using linear regression line. Accuracy and precision was determined by making the sample solution ofloxacin 1, 3, and 5 μg/ml and the internal standard ciprofloxacin 3 μg/ml in blood plasma was extracted using SPE. 10 μl of analyte injected into the HPLC equipment in optimum condition, the experiment was repeated three times, then calculated percent accuracy (recovery) and precision (coefficient of variation). System suitability test conducted on samples Ofloxacin 0.25 μg/ml and the internal standard ciprofloxacin 3 μg/ml in blood plasma, and then extracted using SPE. 10 μl of analyte injected into the HPLC equipment in optimum condition, done six times a repetition then calculated the coefficient of variation of retention time, area ratio and peak height ratio chromatogram.

Results and discussion

Determination of wavelength and molar extinction value

Ofloxacin maximum absorption was obtained at a wavelength of 295 nm and absorption maximum at 279 nm for ciprofloxacin. Ofloxacin maximum wavelength used in the detection analysis by HPLC. The result of the determination of molar extinction ofloxacin showed an average value of 33238.89 M-1cm-1 values>10 000, this shows that it is possible to ofloxacin and detected with UV detector in HPLC systems. This was due to the long chromophore groups in the structure of ofloxacin. Ofloxacin molar extinction values listed in Table 1.

TABLE 1. CALCULATED MOLAR EXTINCTION OF (ε) OFLOXACIN

Ofloxacin extinction molar data in mobile phase* at wavelength of 295 nm			
No.	Molarity (M)	Absorbance	Extinction Molar ε ($M^{-1}cm^{-1}$)
1	0.0000069	0.2231	32.333,33
2	0.0000135	0.4734	35.066,67
3	0.0000180	0.5817	32.316,67
Total			99.716,67
X			33.238,89

Notes: mobile phase of phosphate buffer: acetonitrile (85.5:14.5).

Optimization of HPLC conditions

Optimization of HPLC conditions performed on chromatographic parameters including retention time, resolution or separation (Rs), the theoretical number of copies (N), column efficiency (HETP) of the various variations of composition, and velocity of mobile phase. HPLC conditions optimization results can be seen in Table 2. Efficiency values (N) showed the results of ≥ 2500, this suggested that the sharp peaks produced enough (Harmita, 2006). Mobile phase with a composition of 0.025 M phosphate buffer pH 2.2 and acetonitrile (85,5:14,5) with a flow rate of 1.2 ml/min was chosen because it produces a resolution of 1.77 (≥ 1.5).

Extraction Process and Outcome Recovery Extraction SPE

Conditioning done to clean the impurities (exposure) on the SPE cartridge during storage and to moisten the SPE cartridge. Plasma samples that had been in-spike with ofloxacin and ciprofloxacin incorporated into the SPE cartridge. Laundering conducted in order to

impurities (endogenous substances) in blood plasma could be discarded and not interfere with the analysis by HPLC. In the elution process was expected ofloxacin and ciprofloxacin were left in the SPE cartridge was eluted after washing. Results of the analysis area and peak height chromatogram of ofloxacin in plasma by SPE and the stages of ofloxacin in the mobile phase without the SPE compared. Ofloxacin extraction recovery results can be seen in Table 3. Recovery value had met the requirements of the extraction efficiency, which ranged from 80-120% (Caufield and Stewart, 2002).

TABLE 2. OPTIMIZATION RESULTS OF COMPOSITION AND MOBILE PHASE FLOW VELOCITY

Mobile phase composition (fosfate buffer pH 2.2: asetonitril)	Mobile phase velocity (ml/min)	Retention time ofloxacin (min)	Resolution (R_s)	Theoretical plates (N)	HETP (L/N)
86:14	1.2	14.250	1.88	3.480.81	0.0718
	1.3	12.750	1.83	3.303.59	0.0757
85.5:14.5	1.2	12.533	1.77	3.365.93	0.0743
	1.3	11.375	1.74	3.356.58	0.0745
5:15	1.2	11.767	1.67	3.457.23	0.0723

Notes: L = Coloumn length (mm).

TABLE 3. RECOVERY RESULTS EXTRACTED OFLOXACIN (N = 3)

Based on area ratio					
Recovery ofloxacin (%)			Recovery ciprofloxacin (%)		
Replication	Ofloxacin concentration (µg/ml)		Replication	Ofloxacin concentration (µg/ml)	
	0.25	5		0.25	5
1	109.37	100.53	1	109.80	104.76
2	107.18	101.19	2	114.37	105.92
3	108.49	100.78	3	115.32	103.78
X	108.35	100.83	X	113.16	104.82
CV%	1.02	0.33	CV%	2.61	1.03
Based on area ratio					
Recovery ofloxacin (%)			Recovery ciprofloxacin (%)		
Replication	Ofloxacin concentration (µg/ml)		Replication	Ofloxacin concentration (µg/ml)	
	0.25	5		0.25	5
1	104.58	104.53	1	93.33	107.84
2	95.25	100.81	2	95.52	102.86
3	99.79	100.24	3	95.32	101.58
X	100.21	101.86	X	94.72	104.09
CV%	4.17	2.29	CV%	1.28	3.18

Notes: [a] Tests conducted by the internal standard ciprofloxacin 3 µg/ml.

[b] "Recovery ciprofloxacin (%)" is a recovery value of the concentration of ciprofloxacin with 3 µg/ml at a concentration of ofloxacin 0,25 µg/ml and 5 µg/ml.

Result Analysis Method Validation

To determine the selectivity of the method used, could be seen from the power of separation (resolution) the two peaks (ofloxacin and ciprofloxacin).

Peak seen in Figure 1. Ofloxacin Rt = 11.092 min apart from the top ciprofloxacin Rt 12.533 min with a resolution of Rs = 1.75, according to the requirements for the resolution of> 1.5 (Snyder, et al., 1997). Test results based on retention time reproducibility ofloxacin, the ratio of the area chromatogram, and the chromatogram peak

height ratio gave the value of the coefficient of variation (CV) <10% for the analysis of biological fluid samples (Harmita, 2006). Repeatability test results can be seen in Table 4.

Linear regression line equation with the best correlation coefficient was used to determine the sample concentration was observed Ofloxacin for the accuracy and precision values. Linear regression line equation based on the ratio of area to be used to determine levels of Ofloxacin was y = 0.7687 x + 0.0163 with r = 0.999914. Based on peak height ratios, linear regression line equation used was y = 0.8638 x + 0.0430 with r = 0.999903.

Line equation of the best calibration curve shown in Figure 2 and 3.

LOD and LOQ determined if the absolute concentration of analytes that were analyzed was relatively small as in the biological matrix (Indrayanto, 1994). LOD and LOQ values were calculated based on calibration curves of the equation that had ofloxacin correlation coefficient (r) the best. LOD value of chromatogram area ratio was 0.0227 μg/ml, and based on chromatogram peak height ratio was 0.0241 μg/ml. LOQ value of chromatogram area ratio was 0.0757 μg/ml, and based on chromatogram peak height ratio was 0.0804 μg/ml.

FIGURE 1. OFLOXACIN AND THE STANDARD CHROMATOGRAM CIPROFLOXACIN INTERNAL

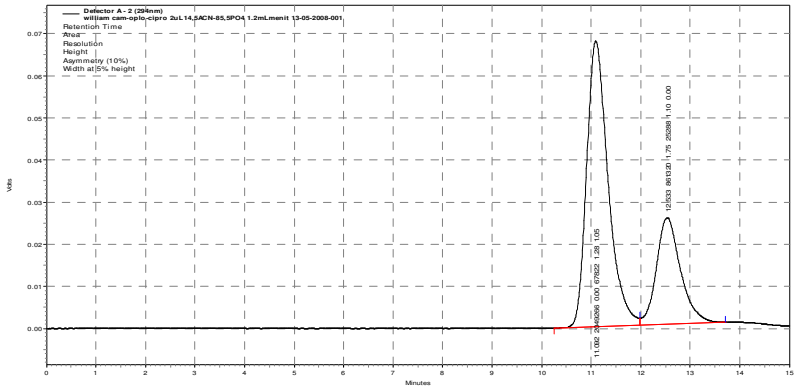
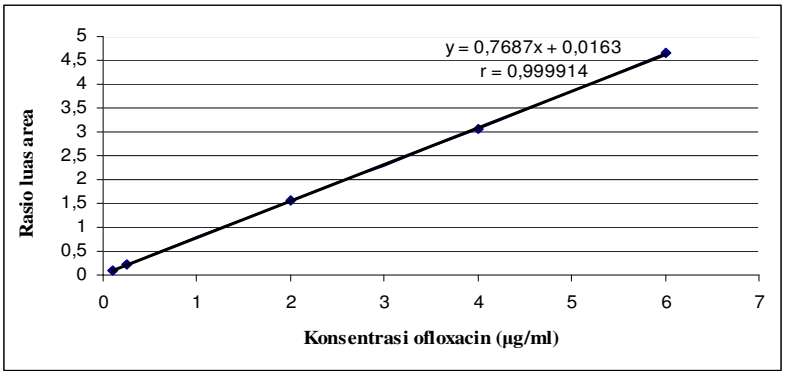


TABLE 4. TEST RESULTS OF REPEATABILITY OFLOXACIN (N = 6)

Concentration ofloxacin (μg/ml)	Retention time ofloxacin	Chromatogram area ratio	Chromatogram height peak ratio
0.25	10.267	0.210585	0.265021
0.25	10.242	0.206751	0.267733
0.25	10.167	0.202026	0.249216
0.25	10.108	0.201882	0.254787
0.25	10.092	0.203039	0.253340
0.25	10.067	0.209726	0.261280
X	10.157	0.205670	0.261280
CV%	0.81	1.90	2.80

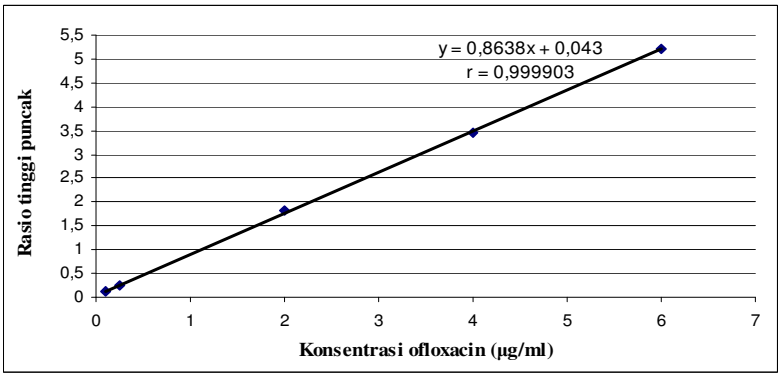
Notes: Test performed in blood plasma with internal standard ciprofloxacin 3 μg/ml

FIGURE 2. OFLOXACIN CALIBRATION CURVE BASED ON THE RATIO OF CHROMATOGRAM AREA



Note: The calibration curve was made from ofloxacin concentrations of 0.10, 0.25, 2.00, 4.00, and 6.00 µg/ml with the internal standard ciprofloxacin 3 µg /ml in blood plasma

FIGURE 2. OFLOXACIN CALIBRATION CURVE BASED ON CHROMATOGRAM PEAK HEIGHT RATIO



Note: The calibration curve was made from ofloxacin concentrations of 0.10, 0.25, 2.00, 4.00, and 6.00 µg / ml with the internal standard ciprofloxacin 3 µg / ml in blood plasma

Based on area ratio and the ratio of chromatographic peak height, the value of accuracy (% recovery) obtained according to the requirements of 80-120% for analysis of biological fluid samples (Harmita, 2006). The precision (% CV) obtained according to the requirements of <10% for the analysis of biological fluid samples (Harmita, 2006). Accuracy and precision of test results can be seen in Table 5.

From the results of system suitability test repeatability demonstrated by injection, indicating that the analytical methods used have met the system suitability criteria CV value of retention time, the ratio of the chromatogram area, and the chromatogram peak height ratio <10% for the analysis of biological fluid samples (Harmita , 2006). With the

value of asymmetry and follow-up factors that meet the requirements of value, ie <2 (LR Snyder et al., 1997). System suitability test results are listed in Table 6.

TABLE 5. TEST RESULT ACCURACY AND PRECISION SAMPLE OFLOXACIN

Based on the chromatogram area ratio			
	Nominal concentration of ofloxacin (µg/ml)		
Replication	1.00	3.00	5.00
	Ofloxacin concentrations obtained (µg/ml)		
1	0.9949	2.8466	4.7700
2	0.9912	2.8297	4.7489
3	1.0045	2.8343	4.8278
	Accuration (%)		
1	99.4884	94.8877	95.3999
2	99.1164	94.3239	94.9775
3	100.4537	94.4779	96.5553
	Precission (% CV)		
	0.69	0.31	0.85
Based on peak height ratio			
	Nominal concentration of ofloxacin (µg/ml)		
Replication	1.00	3.00	5.00
	Ofloxacin concentrations obtained (µg/ml)		
1	1.0013	2.9863	4.8842
2	1.0163	3.0250	4.9389
3	1.0155	2.9609	4.9732
	Accuration (%)		
1	100.1260	99.5445	97.6847
2	101.6263	100.8340	98.7776
3	101.5478	98.6978	99.4636
	Precission (% CV)		
	0.84	1.08	0.91

Notes: a "nominal Ofloxacin Concentration" is a result of spiking Ofloxacin concentrations in blood plasma with internal standard ciprofloxacin 3 tg/ml

b "Ofloxacin concentrations obtained" in the area ratio is calculated Ofloxacin concentration of the standard curve equation y = 0.7687 x +0.0163, r = 0.9999

c "Ofloxacin concentrations obtained" on the peak height ratio is Ofloxacin concentration calculated from standard curve equation y = 0.8638 x +0.0430, r = 0.9999

TABLE 6. SUITABILITY TEST OF OFLOXACIN (N = 6)

CV values based on Retention time, chromatogram area and peak height		Ofloxacin concentration 0,25 µg/ml Variation coefficient values (CV)
Retention time	Ofloxacin	0.0081
	Ciprofloxacin	0.0091
	Ratio	0.0013
Chromatogram's area	Ofloxacin	0.0387
	Ciprofloxacin	0.0322
	Ratio	0.0190
Chromatogram's peak height	Ofloxacin	0.0247
	Ciprofloxacin	0.0107
	Ratio	0.0280
Asymmetry	Ofloxacin	1.06-1.37
	Ciprofloxacin	1.02-1.11
Follow-up factor	Ofloxacin	1.02-1.16
	Ciprofloxacin	1.02-1.05

Notes: Suitability test was performed on Ofloxacin 0.25µg /mL in blood plasma with internal standard ciprofloxacin 3 µg/mL.

Conclusions and recommendations

Optimization of HPLC conditions and ofloxacin extraction from blood plasma using SPE Oasis HLB 1 cc can be done well, so that further analysis can be done by HPLC UV detector. From the results of the validation methods that include parameters: selectivity, repeatability, linearity, detection limit, quantification limit, precision, accuracy, and suitability of the system, the methods used have validity according to the requirements that can be used to analyze Ofloxacin in human blood plasma.

To get better results, it is necessary to do such things as follows: (a), Ofloxacin extraction from blood plasma can be done using different SPE with cartridges that are bound to impurities can be further minimized. (2) Using the volunteers so that the results obtained can be directly used to test the bioavailability and to test its bioequivalence.

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