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Original Article

APPLICATION OFF-LINE SPE-HPLC/UV METHODS IN ANALYSIS OF OFLOXACIN IN HUMAN URINE (*IN VITRO*)

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ABSTRACT

Objective: The objective of this study is to determine the validity of analytical methods in OFX antibiotic study in human urine (*in vitro*) using an SPE-HPLC/UV. In this study, SPE was applied in preparing the analysis of ofloxacin using HPLC embedded UV detector.

Methods: C-18 (octadecylsilane) cartridge (100 mg, particle size 10 µm) of SPE was employed in preparing a sample to determine further of analytes using HPLC with phosphate buffer 0.025 M (pH 2.5) and acetonitrile (85.5:14.5) as mobile phase and a flow rate of 1.2 m l/min. UV detector was adjusted at 295 nm with the internal standard ciprofloxacin.

Results: The calibration curves for the ofloxacin were linear over concentrations ranging from 1.15 to 36.0 µg/ml with a correlation coefficient (r) from 0.9998 to 0.9999. The coefficients of variation obtained from ofloxacin were less than 10 %. Ofloxacin on the area ratio of peak height and a segment of the chromatogram, LOD and LOQ of ofloxacin were 0.12 and 0.4 µg/ml, respectively. The recovery of ofloxacin from spiked human urine was 96.0 %.

Conclusion: The validation methods that including 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 might be used to analyze ofloxacin in human urine.

Keywords: Ofloxacin, Solid Phase Extraction, HPLC.

INTRODUCTION

Sample preparation is an important part of the analysis of drug in a material biologic sample which able to determine the efficacy of analysis because it can establish reproducibility and recovery of the matrix interference [1-4]. The parent metabolite compound (such as protein, carbohydrate, and lipid) should be reduced because the existence of a drug may give misinterpreting results in analysis methods [5]. Therefore, it is needed the method that could accurately identify both the parent drug and metabolites.

Application of SPE (Solid Phase Extraction) in sample preparation can reduce solvent volume and time [2, 6, 7]. In the previous research, application of SPE in the determination of lead compounds aromatherapy in blood plasma of mice after essential inhalation oil obtain good reliability (recovery 90 %) and reproducibility (variation coefficient less than 15 %).

Oflaxocin (OFX) (9-fluoro-2,3-dihydro-3-methyl-10-(-methyl-1-piperzinyl)-7-oxo-7H-pyrido-[1,2,3-de]1,4-bonzoxazine-6-carboxylic acid)as shown fig. 1 is which second generation quinolones are broad spectrum [8-10]. It has activity against both gram-positive and gram-negative bacteria [11]. The OFX inhibits DNA gyrase activity [12].

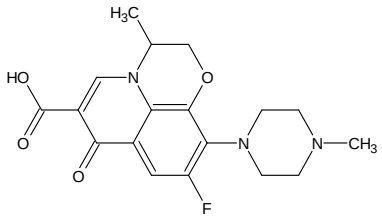

Fig. 1: Structure of ofloxacin

The methods of determination of OFX in human plasma were published by many researchers [13-17] for bioavailability and bioequivalence study. Application of Solid Phase in preparation sample of OFX analysis in human urine has been reported [18-20].

Here, the preliminary study was carried out prior to the clinical study, using human urine. The simple and validated method for measuring the concentration of the OFX in spiked human urine was required in a clinical test.

The aim of this study is to develop a simple and reliable HPLC method for measurement of ofloxacin concentrations in spiked human urine with application SPE for preparation sample. The further recommendation will help in clinical study and routine analysis.

MATERIALS AND METHODS

Chemicals

OFX and ciprofloxacin were purchased from Zhejiang Jinxin, 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), aqua bidest (IPHA), human urine (volunteer, man 20-24 age).

Tools

HPLC (Shimadzu LC-10 ATVP) embedded 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, UV-Vis spectrophotometer (Analytical Jena, specord 200), pH meter (Oh meter), 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 glassware.

Methods

The mobile phase was a mixture of 0.025 M phosphate buffer pH 2.2 and acetonitrile (85:15). The mixture was filtered usin$_g$ 0.45 p. m millipore with vacuum assistance and ultrasonic bath for 15-20 min.

Standard solution preparation

OFX 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 mg/ml. The in-scanning solution with a UV-spectrophotometer at a wavelen$_g$th of 200-320 nm, so the obtained spectrum maximum wavelength (λ max) of absorption and OFX. The same procedure conducted on ciprofloxacin.

Determination of molar extinction

OFX standard solution with a concentration of 6.9, 13.5, and 18.0 pM measured at a wavelength of maximum absorbance OFX, and the calculated values molar extinction.

Optimization of HPLC conditions

OFX standard solution 0.1 mg/ml containin$_g$ the internal standard ciprofloxacin 0.1 mg/ml was injected with 10 p. l (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 (OFX and ciprofloxacin) were produced.

Extraction by solid phase extraction (SPE)

The cartridge of SPE was conditioned by 1 ml of methanol and 1 ml aqua bidest with vacuum assistance. OFX was spiked into plasma with various concentration (0.10, 0.25, 1.00, 2.00, 3.00, 4.00, 5.00, and 6.00 µg/ml). Subsequently, 1 ml of plasma was put into a cartridge of SPE eluted by 1 ml acetonitrile 20% (in phosphate buffer) further injected into the HPLC. The efficiency of SPE extraction was calculated.

Method validation analysis

Selectivity was measured by looking at the chromatogram OFX and ciprofloxacin that obtained from HPLC separation, further calculated the value of the resolution. Repeatability was evaluated by generating a solution of OFX 0.25µg/ml in blood plasma and further extracted by using SPE. 10 µl of analyte was 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 OFX (0.10, 0.25, 2.00, 4.00, and 6.00 µg/ml) and the internal standard ciprofloxacin 3 µg/ml in human urine, further extracted using SPE.

HPLC system embedded with UV (294 nm) to be used as followed column C-18 (octadecyl silane), length of 250 mm, diameter in 4.6 mm, and the particle size of 10 im, mobile phase 0.025 M phosphate buffer pH 2.5 and acetonitrile with ratio of 85.5: 14.5 v/v, and flow rate 1.2 ml/min.

Precision, accuracy, and recovery

Calibration curve equation with the best correlation coefficient was used to specify the sample. LOD and LOQ were determined statistically from the calibration curve equation using linear regression. Accuracy and precision were obtained by making the sample solution OFX 1, 3, and 5 lag/ml and the internal standard ciprofloxacin 3 lag/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 and then calculated percent accuracy (recovery) and precision (coefficient of variation). System suitability test conducted on samples OFX 0.25 lag/ml and the internal standard ciprofloxacin 3µg/ml in blood plasma, and then extracted using SPE. Ten microliters of analyte were 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

The determination of maximum wavelength (λ max)

The result of scanning using UV at the wavelength of 200-380 nm of OFX solutions in the mobile phase (phosphate buffer pH 2.5 and acetonitrile with a ratio of 85.5: 14.5) showed maximum absorption of OFX at λ max of 294 nm. This result was in line with a previous study [21]. Subsequently, the spectrum absorption of ciprofloxacin as a standard was observed. It showed a quite similar λ max to OFX (277 nm). However, the previous study obtained that the λ max of OFX is 275 nm [22] and 278 nm [23].

On the other hand, the result of the combined spectra of both compounds showed the point of intersection at a wavelength of 286.2 nm, as shown in fig. 1.

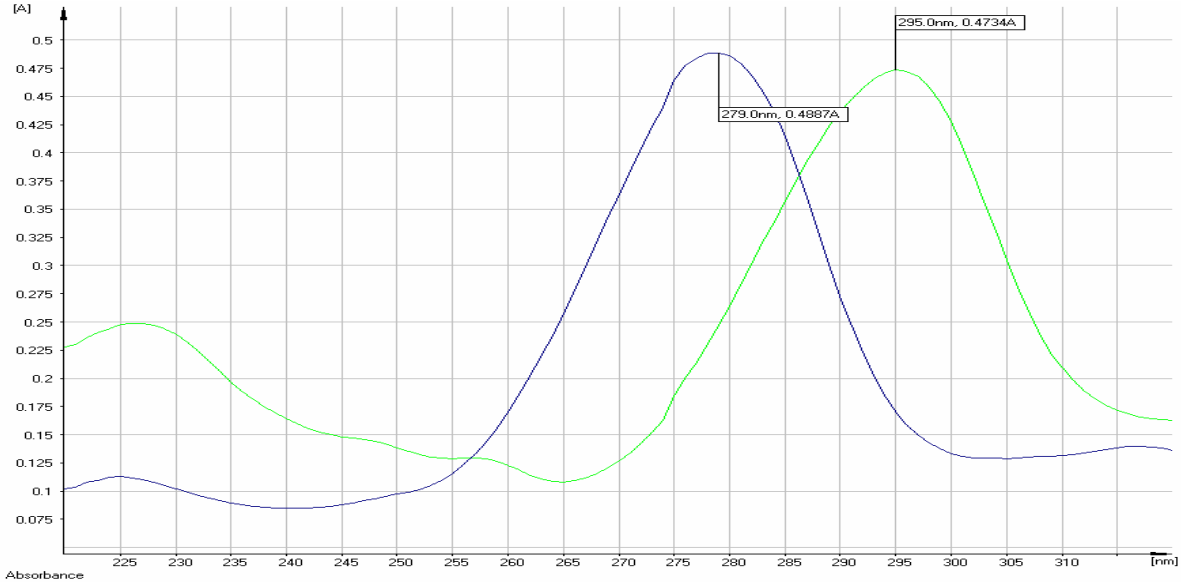

Fig. 1: Spectrum of ciprofloxacin (blue line) was 277 nm dan OFX (green line) was 294 nm

Ciprofloxacin was used as an internal standard due to it has identical chemical structure and properties OFX thus it could be eluted as OFX. The purpose of using internal standard is to reduce errors during the analysis process, particularly, for samples undergoing pre- treatment's, such as extraction, and filtration [24]. y applying the internal standard technique, it was expected to produce sensitive, relatively fast, and accurate method for the analysis of OFX in biological the internal standard technique.

The λ max of OFX was set as the wavelength used in the detection of the analysis result by HPLC, as OFX was the compound of target analysis, ciprofloxacin then provided large absorption at the λ max of OFX.

The determination result of molar extinction value of OFX

The determination of molar extinction value has been conducted to obtain the sensitivity value of OFX. It could be calculated by comparing the absorptivity value or of molar OFX absorptivity towards the thickness of cuvette (usually 1 cm), with the OFX concentration measured[25]. The extinction value of molar OFX from three varied concentrations was 6.9; 13.5; and 18 µM in 0.1 M HCl in a row. It showed that the molar extinction value had an average value of 3.2 $10^4 M^{-1}$ cm^{-1}. This molar extinction value was greater than 10.000 M^{-1} cm^{-1}, indicating that OFX was possible to detect the ultraviolet detector on the HPLC system. The extinction value of OFX could be seen in table 1. The other study measured the extinction value of OFX in 0.1 M HCl at 293 nm and produced 3.5. 10^4 M^{-1} cm^{-1} [26]. However, this result was determined with different diluted concentration.

Table 1: The extinction molar (ε) value of OFX

Extinction molar data OFX in mobile phase at 295 nm			
No.	**Molarity (M)**	**Absorbance**	**Extinction molar ε (M^{-1} cm^{-1})**
1	0.0000069	0.2231	$3.2.10^4$
2	0.0000135	0.4734	$3.5.10^4$
3	0.0000180	0.5817	$3.2.10^4$
Jumlah			$9.9.10^4$
$\overline{X}$			$3,3.10^4$

* Mobile phase = Buffer phosphat: acetonitril (85: 15)

Optimization result of HPLC condition

Optimization result of HPLC condition has been employed chromatography parameters, including the retention time, resolution or separation (Rs), efficiency (N) and column efficiency (HETP) from various compositions. The flow rate of the mobile phase was presented in table 2. The main priority in selecting the method was the resolution value result ≥ 1.5[27]. Based on the Rs value ≥ 1.5, it showed that the two peaks were completely separated. The second priority was the retention time, meaning that the faster retention time was better because the analysis time required will be faster. Efficiency (N) ≥ 2500 showed that the peaks produced were sharp. The theoretical chip value would increase by lowering the flow rate of the mobile phase or by increasing the length of the column, but the analysis time would remain longer.

The mobile phase composed 0.025 M phosphate buffer pH 2.5 and acetonitrile (85: 15) with a flow rate of 1.2 ml/min, it obtained the retention time of OFX in 11.767 min. This condition was in a good state, but there was a disruption of urine that used to be separated in the early minutes of separation. Therefore, it was necessary to examine another mobile phase condition to slow the retention time of OFX.

Furthermore, the observation continued to try a mobile phase composed of 0.025 M phosphate buffer pH 2.5 and acetonitrile (85.5: 14.5) with the flow rate of 1.2 ml/min. The retention time of OFX obtained was 12.533 min while; at the flow rate of 1.3 ml/min, the retention time of OFX obtained was 11.375 min. The retention time of OFX obtained with the mobile phase composed of 0.025 M phosphate buffer pH 2.5 and acetonitrile (86: 14) with a flow rate of 1.2 ml/min is 14,250 min, while at the flow rate of 1.3 ml/min, the retention time of OFX obtained is 12.750 min.

The mobile phase composed of 0.025 M phosphate buffer pH 2.5 and acetonitrile (85.5: 14.5) was chosen, since it produces a good resolution, 1,77 (≥ 1.5). The flow rate of 1.2 ml/min was chosen to optimize the analysis condition because the other slower flow rate would take longer time. The previous study, the mobile phase used in OFX analysis was sodium lauryl sulfate (0.024% aqueous solution)-acetonitrile-glacial acetic acid (500:480:20) [28] or water-acetonitrile-triethylamine (83:14:0.45, v/v, pH 2.30) [29].

The number of theoretical chips (N) in each condition was ≥ 2,500. It indicated that the peak produced was quite sharp [30]. In addition, the number of theoretical chips could be used to determine the quality and performance of the column. The theoretical chip value would rose by lowering the flow rate of the mobile phase or by increasing the length of the column, thereby taking the analysis time much longer.

The result of condition optimization using solid phase extraction (SPE)

The first step of extraction using SPE was conditioned with adding 1 ml of methanol and 1 ml aqua bidest to clean impurities (exposure) in SPE cartridge during storage and also to wet the SPE cartridge [31]. Urine sample spiked by OFX was put in SPE cartridge as much as 1 ml (Sample Loading). The addition of sample has been carried out by drop wise while being energized by negative air pressure using a vacuum to speed up the extraction process and prevent clogged SPE cartridge.

The washing process in SPE phase has been done by adding 1 ml of methanol 3% (in aqua bidest) to clean impurities (endogenous substances) in the urine and conserve the peaks of OFX and ciprofloxacin when analyzed by HPLC. Elution process has been conducted by adding 1 ml of acetonitrile 20% (in 0.025 M phosphate buffer pH 2.5). It was expected that the OFX and ciprofloxacin remained in the SPE cartridge could be eluted or completely pushed out. The output analyte was accommodated in a container, to be analyzed by HPLC.

Elution process was utilizing acetonitrile 20% in 0.025 M phosphate buffer pH 2.5 was sufficient to elute OFX and ciprofloxacin from SPE cartridges. It could be seen from the generated value of the extraction efficiency, which is ≥ 85% for two concentrations, respectively 1.8 and 36 µg/ml. Organic solvents with stronger elution could be used to obtain a better elution result, otherwise using the composition of acetonitrile that is greater than 20%. Due to the composition of the elution containing phosphate buffer pH 2.5 and acetonitrile is in accordance with the composition of the mobile phase of the HPLC system used, the elution result could be directly injected into the HPLC system.

Table 2: Recovery of extraction of OFX 1.8 and 36 µg/ml with internal standard of ciprofloxacin 10 µg/ml based on to area under curve of chromatogram (n = 3)

Recovery of OFX (%)			Recovery of ciprofloxacin (%)		
Replication	**OFX (µg/ml)**		**Replication**	**ciprofloxacin (µg/ml)**	
	1.8	**36**		**1.8**	**36**
1	96.70	99.09	1	92.94	99.50
2	101.41	99.49	2	96.26	91.02
3	101.19	99.21	3	84.06	96.75
$\overline{X}$	99.77	99.27	$\overline{X}$	91.09	95.76
CV	0.0217	0.0017	CV	0.0565	0.0369

To determine the recovery efficiency of the extraction result, the evaluation of extraction efficiency was conducted towards two samples with the concentrations of 1.8 and 36 µg/ml. After that, the injection result in the form of chromatogram area and peak chromatogram height of each sample in the mobile phase without the SPE between the SPE phases in the urine was compared. In table 2, it could be seen that SPE result of the low concentration is 1.8 µg/ml based on the chromatogram area provides 99.77% of the extraction efficiency of OFX and 91.09% of ciprofloxacin while the high concentration of 36 µg/ml is 99.27% and 95.76% respectively.

Extraction result using the SPE as a whole for µg/ml was very satisfying, because of the extraction efficiency is ≥ 85% and the value has met the extraction efficiency requirement>80%[32]. Chromatogram extraction recovery could be seen in fig. 2.

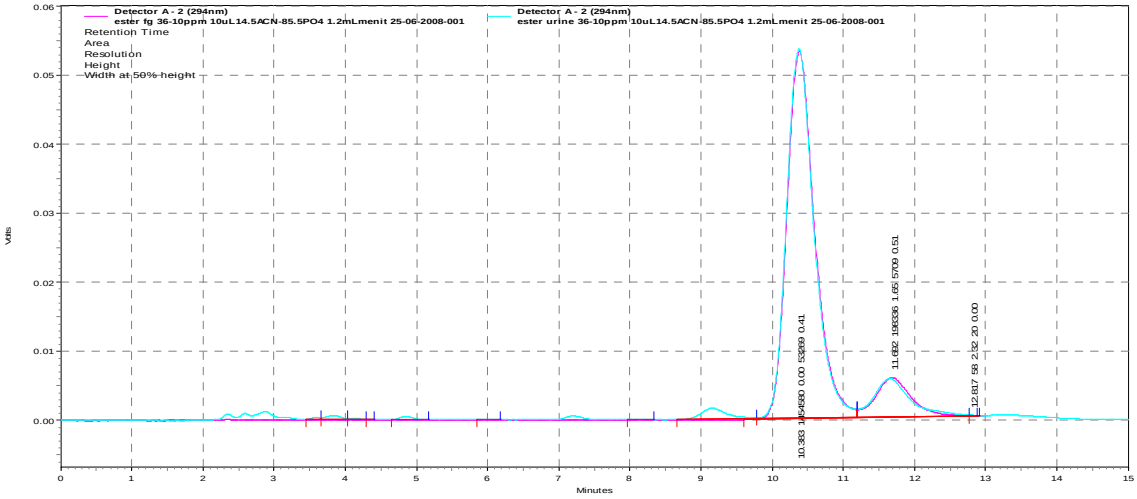

Fig. 2: Chromatogram of *recovery*extraction OFX 36 µg/ml, (purple) in moble phase and (blue) in urine

Selectivity

Selectivity test has been done by observing the resolution indicated at the peak of Rt = 9.867 min for OFX that was perfect apart from the top of ciprofloxacin peak Rt = 11.100 min, with a resolution value of 1.7 exceeding the recommended requirement for a good resolution value, which is ≥ 1.5. According to the resolution value, the HPLC method used had a good selectivity, and could be used to determine the OFX levels by using internal ciprofloxacin standard.

Repeatability tests result

Repeatability test was applied to the OFX sample with a concentration of 1.8 µg/ml, using ciprofloxacin of 10 µg/ml as an internal standard. The replication was performed 6 times, while the retention time, chromatogram area, and high peak chromatogram are observed afterward. The average retention time for OFX was 10.07 min and CV value being<0.67. It indicated a good repeatability value, whereas the required value is<20% for biological fluid sample analysis [27]. The results were presented in table 3.

Table 3: Repeatability analysis results of OFX 1,8 µg/ml with Internal Standard of Ciprofloxacin 10 µg/ml in Urine (n = 6) based on retention time

OFX	Retention time (min)		
(µg/ml)	OFX	Ciprofloxacin	Ratio
1.8	10.042	11.300	0.8886726
1.8	10.025	11.292	0.8877967
1.8	10.200	11.483	0.8882696
1.8	10.025	11.267	0.8897666
1.8	10.092	11.375	0.8872088
1.8	10.058	11.325	0.8881236
$\overline{X}$	10.07	11.34	0.8883063
CV	0.66267	0.69581	0.09769

Table 4: Repeatability analysis results of OFX 1,8 µg/ml with internal standard of ciprofloxacin 10 µg/ml based on area under curve

OFX	Area under curve		
(µg/ml)	OFX	Ciprofloxacin	Ratio
1.8	97501	149397	0.652630
1.8	70535	145583	0.484500
1.8	77786	149236	0.521228
1.8	80065	152391	0.525392
1.8	80121	146883	0.545475
1.8	79496	147747	0.538055
$\overline{X}$	80917.33	148539.5	0.54454673
CV	0.10998	0.01598	0.10466

The repeatability results were good for the CV was<0.11 (table 4), while the required value is<0.2 for the analysis of biological fluid sample[33]. Likewise, the result of repeatability based on chromatogram peak height ratio indicated a good value with a coefficient of variation (CV)<0.05 (table 4).

In particular literature, strict criteria were obtained if the method provides relative standard deviation, or 2% or less of CV. However, this value was very flexible depending on the concentration of the analyte analyzed, the number of samples, and laboratory condition. The variation coefficient would increase as the analyte level analyzed decrease [27, 33, 34]. The data proved that the method used has a good repeatability, with CV value <20% for the analysis of biological fluid sample[27, 34].

Linearity result

Linearity test was conducted to observe the capability of analytical method in giving a good response to various analyte concentrations on a calibration curve in order to produce a straight line. The parameter concerning linear relationship was expressed by the correlation coefficient and a valid analytical method which has a correlation coefficient more than 0.998[33].

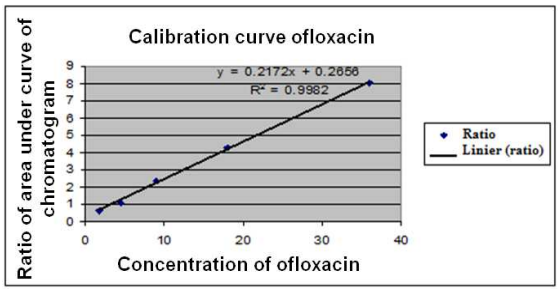

Fig. 3: Calibration curve of OFX with ranged concentration (1,8-36 µg/ml) that obtained internal standard of ciprofloxacin 10 µg/ml based on ratio of Area Under Curve of chromatogram

OFX extraction result of ciprofloxacin in the urine was ranged from 1.8-36 µg/ml obtaining a linear calibration curve with the line equation y = 0,2172x+0.2656 and the correlation coefficient (R) = 0.9982, containing ciprofloxacin internal standard 10 µg/ml based on the ratio of chromatogram area (fig. 3).

Limit of detection (LOD) and limit of quantitation (LOQ)

The absolute limit of detection (LOD) was determined when the concentration of the analyte analyzed was relatively small as in biological matrix [30, 35]. The result of the limit of detection (LOD) test was calculated based on the calibration curve from an equation with the best correlation coefficient (r). The LOD value was depending on the calibration curve of OFX towards the ratio of the chromatogram area[29]. The LOD value of the area ratio was 0.12 µg/ml.

The absolute limit of quantitation (LOQ) is determined when the concentration of the analyte analyzed relatively small as in biological matrix [30, 35]. The result of the limit of quantitation (LOQ) test was calculated based on the calibration curve of OFX in accordance with an equation that had the best correlation coefficient (r). LOQ value was determined from the calibration curve of OFX towards the ratio of chromatogram area. LOQ value of the area ratio was 0.4 µg/ml.

Precision and accuracy

According to the calculation of the sample levels based on chromatogram area, it provided precision values expressed as CV from concentrations of 1.8 CV; 9 and 36 µg/ml, 0.16; 0,025; and 0.038, respectively. The value was fair as required (<20%) for the analysis of biological fluid sample [30]. While the accuracy values in % sample recovery with concentrations of 1.8; 9 and 36 µg/ml were 80.09%; 109.89% and 90.01%, respectively. The values obtained were fairly good as required as 80-110% [33].

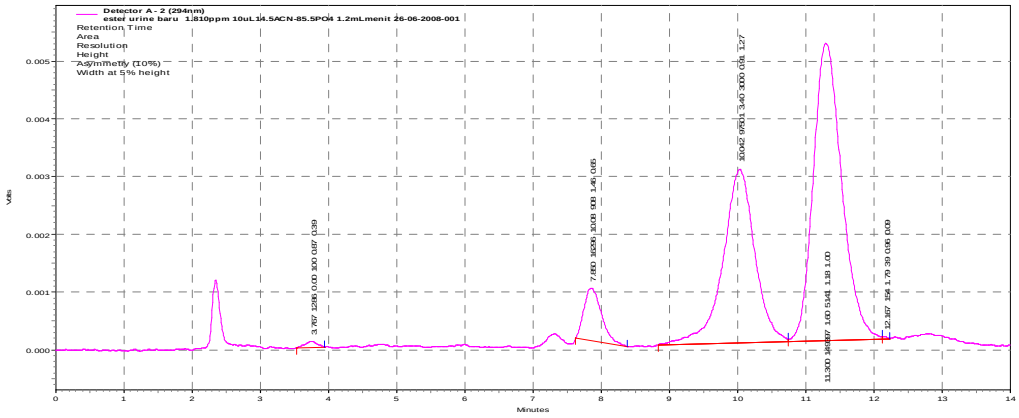

Fig. 4: Chromatogram in system suitability of OFX 1.8 µg/ml with internal standard ciprofloxacin 10 µg/ml in human urine

Table 5: System suitability analysis of OFX in concentration 1.8 µg/ml with internal standard of ciprofloxacin 10 µg/ml in human urine (n = 6)

Parameters		OFXIn 1.8 µg/ml
		Variation coefficient (CV)
Retention time	OFX	0,66267
	Ciprofloxacin	0,69581
	Ratio	0,09769
Area under curve	OFX	0,10998
	Ciprofloxacin	0,01598
	Ratio	0,10466
Peak height	OFX	0,04592
	Ciprofloxacin	0,00088
	Ratio	0,04492
Asymmetry	OFX	1,06-1,37
	Ciprofloxacin	1,02-1,11
Tailing factor	OFX	1,02-1,16
	Ciprofloxacin	1,02-1,05

System suitability

Table 5 showed that the Consistent Variation (CV) from the retention time, the ratio of the chromatogram area, and the chromatogram peak height ratio ≤ 10% for the analysis of the biological fluid sample. It indicated that the method used had good system suitability.

The suitability of the system was also used to determine the asymmetry factor and tailing factor of the peak. The asymmetry and tailing factors were used to determine the column condition and the experimental condition. The OFX peak of asymmetry with a concentration of 1.8 µg/ml was equal to 0.91-1.04 while the ciprofloxacin was equal to 1.02-1.11. The asymmetry value has met the criteria of the<2[27]. The tailing factor value for the peak of OFX with a concentration of 1.8 µg/ml was equal to 1.02 to 1.16 and for ciprofloxacin was equal to 1.02 to 1.05. The tailing factors obtained have met the requirements value, which was < 2 (the chromatogram could be seen in fig. 4).

CONCLUSION

OFX in human urine could be extracted by using SPE Oasis HLB 1 thus further analysis by HPLC embedded UV detector with recovery more than 96 %. The validation methods that including parameters: selectivity, repeatability, linearity, detection limit, quantification limit, precision, accuracy, and suitability of the system, the methods used were valid according to the requirements that might be used to analyze OFX in human urine.

ABBREVIATION

HPLC/UV: High-Performance Liquid Chromatography-Ultra Violet, SPE: Solid Phase Extraction, LOD: Limit of Detection, LOQ: Limit of Quantification, OFX: ofloxacin.

CONFLICT OF INTERESTS

Declared none

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