

The development of HPLC dual wavelength fluorescence detection method for the simultaneous determination of four fluoroquinolones in human plasma

Diep Thi Minh Nguyen, Tuan Anh Vu, Dien Xuan Luong, Truong Xuan Nguyen*

School of Chemical Engineering, Hanoi University of Science and Technology,
1 Dai Co Viet Street, Bach Khoa Ward, Hai Ba Trung District, Hanoi, Vietnam

Received 31 May 2021; revised 10 July 2021; accepted 20 July 2021

Abstract:

A high-performance liquid chromatography-fluorescence detection (HPLC-FLD) method for determination of the fluoroquinolones norfloxacin (NOR), ciprofloxacin (CIP), levofloxacin (LEVO), and moxifloxacin (MOXI) in human plasma has been successfully developed. The detection was optimized with a dual wavelength excitation and emission fluorescence detector. Analytical wavelengths of $\lambda_{ex}/\lambda_{em} = 290/500$ nm (for LEVO and MOXI) and $\lambda_{ex}/\lambda_{em} = 280/445$ nm (for NOR and CIP) were found when measuring fluoroquinolones in the mixture. The plasma sample was pre-treated during the deproteinization step using methanol. This method was validated by linearity in the ranges of 0.05-15; 0.05-10; 0.05-15; and 0.10-10 $\mu\text{g ml}^{-1}$ with limits of detection of 0.0071; 0.0080; 0.0074; and 0.0171 $\mu\text{g ml}^{-1}$ for NOR, CIP, LEVO, and MOXI, respectively. Further, the HPLC-FLD method was proven to be precise and accurate with relative standard deviations lower than 6% and recoveries ranging from 92.5-105.4% for all four fluoroquinolones. Therefore, the proposed HPLC-FLD method provides an alternative approach for the simultaneous analysis of fluoroquinolones in plasma.

Keywords: dual wavelength excitation and emission, fluoroquinolones, HPLC-FLD, plasma.

Classification number: 2.1

1. Introduction

Fluoroquinolones (FQs) are an important and effective class of antibacterial agents. Indeed, FQs have broad-spectrum antibacterial activity, long elimination half-lives, and good tissue penetration. To improve bactericidal activity and pharmacological properties, numerous fluoroquinolones have been developed. Currently, they are classified as second-, third-, and fourth-generation quinolones and are indicated for treatment of a large variety of infections such as respiratory, gastrointestinal, and gynaecologic, as well as sexually transmitted diseases, prostatitis, and infections of the skin, soft tissue, bone, and joints [1-3].

The appropriate use of these fluoroquinolones is an important issue that remains a difficult challenge. One approach is to continuously monitor pharmacokinetic behaviour and activity of an antibiotic in changing therapeutic environments in order to select the optimal agent, its dose, and duration of treatment so that therapeutic failure and/or the development of antibacterial

resistance can be avoided [4, 5]. On the other hand, it has been thought the administration of a combination of FQs may result in a superior therapeutic outcome than that of an individual FQ. To verify this, HPLC with fluorescence detection (FLD), one the most sensitive methods for the determination of fluoroquinolones in plasma, is employed [6-8]. It is common in HPLC-FLD to fix the excitation and emission wavelengths. For example, the detection of norfloxacin, ciprofloxacin, levofloxacin, or moxifloxacin is shown in Table 1. In a similar manner, the simultaneous detection of two FQs: ciprofloxacin and moxifloxacin [9], and levofloxacin and moxifloxacin [10], or three FQs: pazufloxacin, ciprofloxacin, and levofloxacin [11], and levofloxacin, gatifloxacin, and moxifloxacin [12], or even five FQs: levofloxacin, pazufloxacin, gatifloxacin, moxifloxacin, and trovafloxacin [13] have been reported. However, the sensitivity of this method was high for certain FQs but low for others. To solve this problem, A.E. Mansilla, et al. (2005) [14] developed photo-induced fluorimetric detection and multi-emission scanning,

*Corresponding author: Email: truong.nguyensexuan@hust.edu.vn

Table 1. Fluoroquinolones determination in plasma by HPLC-FLD.

Analysed compound	Sample preparation	Mobile phase	Ex/Em wavelength	LOD/LOQ $\mu\text{g ml}^{-1}$	References
NOR	PP with acetonitrile	0.015 mol l ⁻¹ sodium heptanesulfonate, 0.2% triethylamine and phosphoric acid (pH 2.5)-acetonitrile-methanol 70:15:15 (v/v/v)	268/445	LOD 0.0059 LOQ 0.0195	[16]
CIP	PP with acetonitrile	phosphoric acid (0.025M)/methanol/acetonitrile (75/13/12%, v/v/v); pH was adjusted to 3.0 by triethylamine	278/450	LOD 0.010 LLOQ 0.020	[17]
LEVO	PP with acetonitrile	acetonitrile and 0.4% triethylamine (pH 3.0) 24:76 (v/v); pH was adjusted to 3.0 by 85% phosphoric acid	295/490	LOD 0.030 LLOQ 0.150	[18]
MOXI	filtration using 0.2 μm syringe filter	acetonitrile and 0.25 mol l ⁻¹ Na ₃ PO ₄ (pH 3.0) 5:95 (v/v); pH was adjusted by phosphoric acid	290/500	LOD 0.001 LOQ 0.003	[19]

PP: protein precipitation; LOD: limit of detection; LOQ: limit of quantification; LLOQ: lower limit of quantification.

which was adequate for the simultaneous determination of the four fluoroquinolones due to fixed excitation at 277 nm. As another solution, a fluorescence detector developed by J.D. Smet, et al. (2009) [15] was programmed to turn the initial excitation/emission wavelengths of 290/500 nm (for ofloxacin, moxifloxacin, sarafloxacin) into those of 279/442 nm (ciprofloxacin) between 5.8 and 8.5 min. However, this method was only suitable for FQs with retention times that were separated by enough time.

Therefore, the purpose of this study is to develop a simple and sensitive HPLC-FLD method for the simultaneous determination of fluoroquinolones in human plasma, which can be used as a model to probe pharmacokinetic behaviour and for therapeutic drug monitoring. The possibility of using dual wavelength excitation and emission allows the selection of appropriate excitation and emission wavelengths, which increases the sensitivity analysis for each FQ (norfloxacin, ciprofloxacin, levofloxacin, and moxifloxacin). To the best of our knowledge, this is the first report of an HPLC-FLD method using two couples of excitation and emission wavelengths for the simultaneous determination of four fluoroquinolones in human plasma.

2. Materials and methods

2.1. Chemicals and reagents

The fluoroquinolone standards norfloxacin (NOR, lot no. QT068050316, 99.5%), ciprofloxacin (CIP, lot

no. QT012120318, 98.3%), levofloxacin (LEVO, lot no. QT165080417, 98.2%), and moxifloxacin (MOXI, lot no. QT187041117, 97.0%) were purchased from the Institute of Drug Quality Control, Ho Chi Minh city, Vietnam. Solvents were HPLC grade (Merck, Germany) and included methanol (MeOH), acetonitrile (ACN), and triethylamine (TEA). The ACS reagents of phosphoric acid (H₃PO₄) and hydrochloric acid (HCl) were purchased from Acros Organics (USA). Deionized (DI) water (18 M Ω cm⁻¹) was obtained with a Millipore Milli-Q water purification system.

2.2. Standard and sample preparation

Standard solutions: the 1000- $\mu\text{g ml}^{-1}$ primary stock standards of each antibiotic, NOR, CIP, LEVO, and MOXI, were prepared in MeOH/0.1 M HCl 1:1 (v:v). Working standard solutions of 100 $\mu\text{g ml}^{-1}$, 10 $\mu\text{g ml}^{-1}$, and 1 $\mu\text{g ml}^{-1}$ were prepared by appropriately diluting the stock solution with methanol. From these solutions, working standard mixtures from each antibiotic were prepared and stored at -20°C for 30 d.

Sample preparation: Blank plasma samples were collected from healthy, drug-free volunteers at a general clinic in Phu Tho province, Vietnam. The spiked sample was prepared by taking a known volume of a standard mixture solution, evaporating the solvent to collect the residue (temperature 40°C, N₂ gas), diluting the residue in the blank plasma, and vortexing it for 5 min to obtain a spiked sample of known concentration. The blank plasma and spiked samples were stored at -20°C for less than one month and were thawed immediately to room temperature before the assay treatment.

Plasma treatment: To 1 ml of thawed plasma, 4 ml of methanol was added to precipitate proteins, then the mixture was vortexed for 5 min and centrifuged for 20 min at 6000 rpm. The supernatant was filtered through 0.45 μm nylon filters before analysis [8].

2.3. Instrumentation

2.3.1. Fluorescent measurement

Fluorescent measurements were performed with a Nanolog spectrofluorometer (Horiba, USA) equipped with 450 W Xe arc lamp and double excitation monochromators. The excitation and emission spectra were recorded automatically during the measurements.

2.3.2. Chromatographic system

Chromatographic analysis was performed on an HPLC system equipped with a Sil-20AC XR automatic sampler, a CTO-20A thermostatic column box, and a RF-20A fluorescent detector (Shimadzu, Japan). The separation was achieved on a Supelco Ascentis C18 column (250×4.6 mm, 5 µm). The mobile phase was a mixture of 0.025 M phosphoric acid (pH 3, adjusted with TEA) aqueous solution (mobile phase A) and MeOH (mobile phase B). The following gradient program (with respect to mobile phase B) was used: 0-2.0 min, 15% B; 2.0-17.0 min, 15-35% B; 17.0-22.0 min, 35-80% B; and 22.0-25.0 min, 80% B. The flow rate was 1.0 ml min⁻¹. The injection volume was 10 µl. The column oven was maintained at 30°C (see Supplementary Information for optimization of the mobile phase and the gradient elution).

2.4. Method development

Detection: To develop a sensitive HPLC method using a dual wavelength excitation and emission fluorescence detector for the simultaneous quantification of the four FQs (NOR, CIP, LEVO and MOXI), the excitation spectrum of each antibiotic was recorded using the Nanolog spectrofluorimeter. Then, a test solution of the mixture was recorded using the HPLC-FLD system at two selected couples of excitation and emission wavelengths.

2.5. Method validation

Method validation was carried out according to the ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) and the AOAC (Association of Official Analytical Chemists) guidelines [20, 21]. The HPLC dual wavelength fluorescence detection method was validated for selectivity, sensitivity, linearity, precision, and accuracy.

Selectivity was assessed by examining peak interference from a blank plasma sample and a spiked sample of the mixture at a concentration of 1 µg ml⁻¹ of each drug.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by gradually reducing the standard mixture of each drug (0.05 µg ml⁻¹) to a blank plasma sample. LOD was obtained at a concentration in

which the height of the peak signal-to-noise (S/N) was equal to 3. LOQ was accepted as $LOQ = 3.3 \times LOD$. From these LOD and LOQ values, the method detection limit (MDL) and quantification limit (MQL) values were estimated by taking a dilution factor of 5 into account during the plasma sample treatment.

Linearity: The calibration samples were prepared in blank plasma over a range of 0.05-20 µg ml⁻¹ (e.g., 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, and 20.0 µg ml⁻¹). The standard calibration curve was constructed by least squares linear regression using peak areas. The linearities of the standard curves were assessed by its intercept, slope, and correlation coefficient (R^2).

Precision and accuracy: The precision and accuracy of the method were evaluated using three quality control (QC) spiked plasma samples: 1.0, 5.0, and 10.0 µg ml⁻¹. The precision was expressed as the relative standard deviation (RSD%) of the measured QC samples in sextuplicate. The accuracy was determined as a relative error bias. The results were calculated from the calibration curves by means of the external standard method. The procedure for sample preparation was the same as described above.

3. Results and discussion

3.1. Method development

Optimization of the detection: Fig. 1 shows the emission and excitation spectra of each antibiotic. The spectra were in accordance with previous studies [9-19], and the maximum excitation wavelengths of the FQs were divided into two groups: approximately 280 nm for NOR and CIP and 290 nm for LEVO and MOXI. Moreover, the fluorescent intensity of NOR or CIP was low if the antibiotic was excited at a wavelength of 290 nm. Chromatograms of the mixture recorded with dual wavelength excitation and emission fluorescence detector are presented in Fig. 2. It was quickly realized that the detection sensitivity was significantly enhanced at the excitation of 280 nm ($\lambda_{em}=445$ nm) for NOR and CIP, while the same occurred at 290 nm for LEVO and MOXI. Hence, the dual channels of the fluorescence detector were optimized at $\lambda_{ex}/\lambda_{em}=290/500$ nm (LEVO and MOXI) and $\lambda_{ex}/\lambda_{em}=280/445$ nm (NOR and CIP) for HPLC-FLD analysis of the FQs in the mixture.

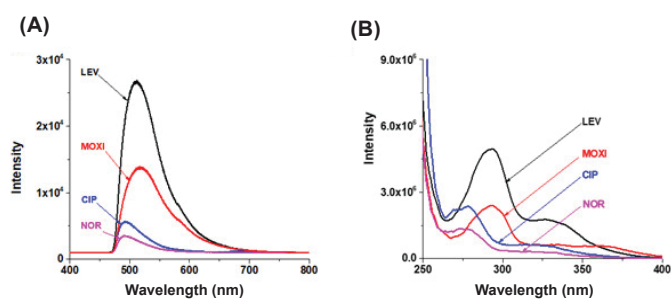


Fig. 1. (A) Emission ($\lambda_{ex}=290$ nm) and (B) excitation ($\lambda_{em}=500$ nm) spectra of each FQ at $1.0 \mu\text{g ml}^{-1}$.

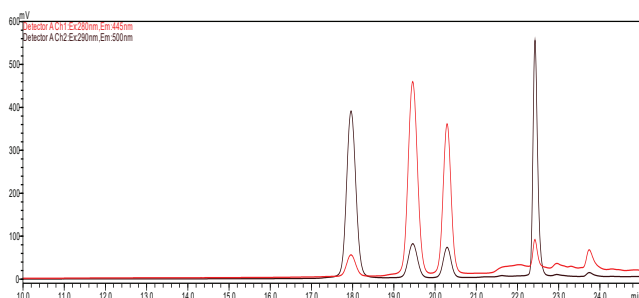


Fig. 2. Chromatograms of the FQ mixture ($0.5 \mu\text{g ml}^{-1}$) recorded with $\lambda_{ex}/\lambda_{em}=290/500$ nm (black curve) and $\lambda_{ex}/\lambda_{em}=280/445$ nm (red curve).

3.2. Method validation

Assay selectivity: Blank plasma samples were analysed and no interference was observed at the retention times of LEVO, NOR, CIP, or MOXI. Representative chromatograms of the blank plasma and spiked samples are shown in Fig. 3.

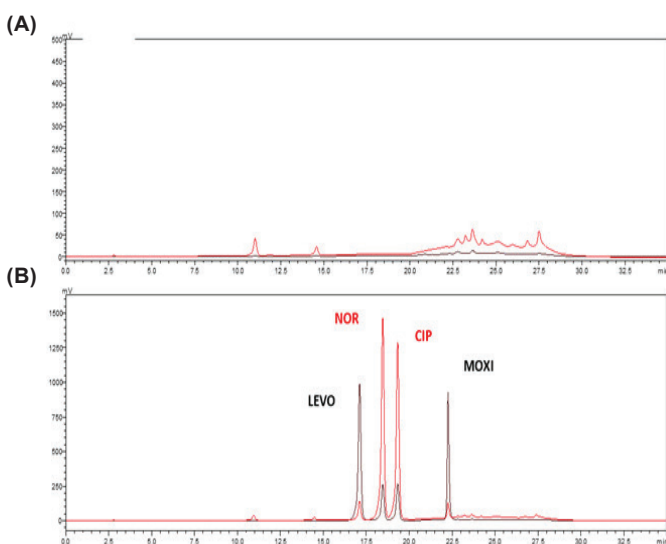


Fig. 3. Chromatograms of (A) the blank plasma sample and (B) the spiked sample ($1.0 \mu\text{g ml}^{-1}$) in plasma recorded with $\lambda_{ex}/\lambda_{em}=290/500$ nm (black curve) and $\lambda_{ex}/\lambda_{em}=280/445$ nm (red curve).

The LOD and LOQ values for the determination of the four FQs are listed in Table 2. It was found that the values for CIP determination in the simultaneous analysis by the developed method were lower than those by the reported method of which its detection ($\lambda_{ex}/\lambda_{em}=292/525$ nm) was not optimized [9]. Therefore, there is a possibility that using appropriate analytical wavelengths improved the sensitivity of the HPLC-FLD method.

Table 2. Validation parameters of the method for the analysis of fluoroquinolones in plasma.

FQs	Regression equation	R ²	Linearity ($\mu\text{g ml}^{-1}$)	LOD ($\mu\text{g ml}^{-1}$)	LOQ ($\mu\text{g ml}^{-1}$)	MDL ($\mu\text{g ml}^{-1}$)	MQL ($\mu\text{g ml}^{-1}$)
NOR	$y = 1.09 \times 10^5 x + 1.50 \times 10^6$	0.999	0.05-15	0.0071	0.0234	0.0355	0.1170
CIP	$y = 1.13 \times 10^5 x + 1.40 \times 10^6$	0.999	0.05-10	0.0080	0.0264	0.0400	0.1320
LEVO	$y = 7.62 \times 10^4 x + 9.70 \times 10^5$	0.999	0.05-15	0.0074	0.0244	0.0370	0.1220
MOXI	$y = 7.30 \times 10^4 x + 1.25 \times 10^6$	0.999	0.10-10	0.0171	0.0564	0.0855	0.2820

y: peak area; x: plasma concentration of the FQ; R²: correlation coefficient; MDL = 5×LOD; MQL = 5×LOQ.

The linearities of the four fluoroquinolones in human plasma by the proposed method were evaluated. The calibration curves were linear over the range $0.05\text{--}15 \mu\text{g ml}^{-1}$ for NOR and LEVO and $0.05\text{--}10 \mu\text{g ml}^{-1}$ for CIP and MOXI (Fig. 4). The linear regression equations with corresponding correlation coefficients of the calibration curves are indicated in Table 2.

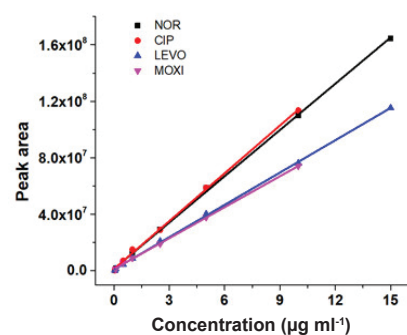


Fig. 4. Calibration curves for determination of the FQs in plasma.

Precision and accuracy: The precision and accuracy data of the analytical method are summarized in Table 3. For all four FQs, the precision was better than 6% at all three concentration levels, and the accuracies ranged from 92.5 to 105.4%. These results indicate that the proposed assay was precise and accurate.

Table 3. Precision and accuracy data of the method for the analysis of the fluoroquinolones in plasma.

FQs	Added conc. $\mu\text{g ml}^{-1}$	Found conc. $\mu\text{g ml}^{-1}$	RSD % (n=6)	Recovery % (n=6)
NOR	1.000	1.010 $\pm$ 0.021	2.1	101.0
	5.000	5.175 $\pm$ 0.075	1.4	103.5
	10.000	9.880 $\pm$ 0.420	4.3	98.8
CIP	1.000	0.950 $\pm$ 0.050	5.3	95.0
	5.000	5.040 $\pm$ 0.072	1.4	100.8
	10.000	10.540 $\pm$ 0.310	2.9	105.4
LEVO	1.000	1.050 $\pm$ 0.018	1.7	105.0
	5.000	4.980 $\pm$ 0.065	1.3	99.6
	10.000	9.965 $\pm$ 0.171	1.7	99.7
MOXI	1.000	0.925 $\pm$ 0.050	5.4	92.5
	5.000	4.990 $\pm$ 0.034	0.68	99.8
	10.000	9.750 $\pm$ 0.272	2.8	97.5

The developed method is suitable for the simultaneous determination of four FQs in human plasma because their peak plasma concentrations were in the ranges of 1.60–2.87 $\mu\text{g ml}^{-1}$ for a single oral administration of norfloxacin 400 mg [22], 1.3–2.0 $\mu\text{g ml}^{-1}$ for an oral administration of ciprofloxacin of 10 mg/kg every 12 h in children with severe malnutrition, 2.8 and 5.2 $\mu\text{g ml}^{-1}$ for 250 and 500 mg oral administration of levofloxacin, respectively [23], and 2.60–3.79 $\mu\text{g ml}^{-1}$ for 400 mg oral administration of moxifloxacin [24].

4. Conclusions

The developed and validated HPLC-FLD method is readily available for the simultaneous determination of the fluoroquinolones norfloxacin, ciprofloxacin, levofloxacin, and moxifloxacin in human plasma. The possibility of using a dual wavelength excitation and emission fluorescence detector avoids the reduced sensitivity of certain FQs in the mixture as detected by other methods. This proposed method enables simple and sensitive quantification of representative fluoroquinolones (second-, third-, and fourth-generation quinolones) in a range from 0.05 to 10 (15) $\mu\text{g ml}^{-1}$ with acceptable precision and accuracy. Although the applicability of the proposed method for analysing real samples requires further investigation, this work contributes an alternative HPLC-FLD approach for therapeutic drug monitoring and pharmacokinetic study.

CRedit author statement

Diep Thi Minh Nguyen, Tuan Anh Vu, Dien Xuan Luong, Truong Xuan Nguyen: Data curation, Software, Visualisation, Investigation, Writing, Methodology, Supervision.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] V.T. Andriole (2005), "The Quinolones: Past, present, and future", *Clinical Infectious Diseases*, **41**(2), pp.113–119, DOI: 10.1086/428051.
 [2] P.C. Sharma, A. Jain, S. Jain (2009), "Fluoroquinolone antibacterials: A review on chemistry, microbiology and therapeutic prospects", *Acta Poloniae Pharmaceutica*, **66**(6), pp.587–604.
 [3] C. Oliphant, G.M. Green (2002), "Quinolones: A comprehensive review", *American Family Physician*, **65**, pp.455–464.

[4] A.M. Emmerson, A.M. Jones (2003), "The quinolones: Decades of development and use", *Journal of Antimicrobial Chemotherapy*, **51**(1), pp.13–20, DOI: 10.1093/jac/dkg2083.
 [5] W.M. Scheld (2003), "Maintaining fluoroquinolone class efficacy: Review of influencing factors", *Emerging Infectious Diseases*, **9**(1), pp.1–9, DOI: 10.3201/eid0901.020277.
 [6] H. Liang, M.B. Kays, K.M. Sowinski (2002), "Trovafoxacin and cinoxacin by high-performance liquid chromatography: Application to levofloxacin determination in human plasma", *Journal of Chromatography B*, **772**(1), pp.53–63, DOI: 10.1016/S1570-0232(02)00046-6.
 [7] J. Sousa, G. Alves, A. Fortuna, et al. (2012), "Analytical methods for determination of new fluoroquinolones in biological matrices and pharmaceutical formulations by liquid chromatography: A review", *Analytical and Bioanalytical Chemistry*, **403**, pp.93–129, DOI: 10.1007/s00216-011-5706-8.
 [8] A. Czyrski (2017), "Analytical methods for determining third and fourth generation fluoroquinolones: A review", *Chromatographia*, **80**, pp.181–200, DOI: 10.1007/s10337-016-3224-8.
 [9] N.C. Rocha, I.C. Alvarado, L.V. Cabrera, et al. (2014), "HPLC method for the simultaneous analysis of fluoroquinolones and oxazolidinones in plasma", *Journal of Chromatographic Science*, **52**(10), pp.1281–1287, DOI: 10.1093/chromsci/bmu002.
 [10] S. Schulte, T. Ackermann, N. Bertram, et al. (2006), "Determination of the newer quinolones levofloxacin and moxifloxacin in plasma by high-performance liquid chromatography with fluorescence detection", *Journal of Chromatographic Science*, **44**(4), pp.205–208, DOI: 10.1093/chromsci/44.4.205.
 [11] S. Watabe, Y. Yokoyama, K. Nakazawa, et al. (2010), "Simultaneous measurement of pazufloxacin, ciprofloxacin, and levofloxacin in human serum by high-performance liquid chromatography with fluorescence detection", *Journal of Chromatography B*, **878**(19), pp.1555–1561, DOI: 10.1016/j.jchromb.2010.04.012.
 [12] J. Grellet, J. Grellet, B.B. Ba, et al. (2004), "Simultaneous determination of levofloxacin, gati-floxacin and moxifloxacin in serum by liquid chromatography with column switching", *Journal of Chromatography B*, **810**(1), pp.77–83, DOI: 10.1016/j.jchromb.2004.07.019.
 [13] J. Sousa, G. Alves, G. Campos, et al. (2013), "First liquid chromatography method for the simultaneous determination of levofloxacin, pazufloxacin, gatifloxacin, moxifloxacin and trovafoxacin in human plasma", *Journal of Chromatography B*, **930**(1), pp.104–111, DOI: 10.1016/j.jchromb.2013.04.036.
 [14] A.E. Mansilla, A.M.D.L. Peña, D.G. Gómez, et al. (2005), "HPLC determination of enoxacin, ciprofloxacin, norfloxacin and ofloxacin with photoinduced fluorimetric (PIF) detection and multiemission scanning", *Journal of Chromatography B*, **882**(1–2), pp.185–193, DOI: 10.1016/j.jchromb.2005.05.045.
 [15] J.D. Smet, K. Boussey, K. Colpaert, et al. (2009), "Pharmacokinetics of fluoroquinolones in critical care patients: A bio-analytical HPLC method for the simultaneous quantification of ofloxacin, ciprofloxacin and moxifloxacin in human plasma", *Journal of Chromatography B*, **877**(10), pp.961–967, DOI: 10.1016/j.jchromb.2009.02.039.
 [16] T. Galaon, S. Udrescu, I. Sora, et al. (2006), "High-throughput liquid-chromatography method with fluorescence detection for reciprocal determination of furosemide or norfloxacin in human plasma", *Biomedical Chromatography*, **21**(1), DOI: 10.1002/bmc.715.
 [17] S.N. Muchohi, N. Thuo, J. Karisa, et al. (2011), "Determination of ciprofloxacin in human plasma using high-performance liquid chromatography coupled with fluorescence detection: Application to a population pharmacokinetics study in children with severe malnutrition", *Journal of Chromatography B*, **879**(2), pp.146–152, DOI: 10.1016/j.jchromb.2010.11.032.
 [18] A. Czyrski, E. Szalek (2016), "An HPLC method for levofloxacin determination and its application in biomedical analysis", *Journal of Analytical Chemistry*, **71**, pp.840–843, DOI: 10.1134/S1061934816080049.
 [19] A.L. Djurdjevic, M.J. Stankov, P. Djurdjevic (2006), "Optimization and validation of the direct HPLC method for the determination of moxifloxacin in plasma", *Journal of Chromatography B*, **844**(1), pp.104–111, DOI: 10.1016/j.jchromb.2006.07.001.
 [20] J. Szpylka, N. Thiex, B. Acevedo, et al. (2019), "Standard method performance requirements (SM- PRs®) 2018.001: Sugars in animal feed, pet food, and human food", *Journal of AOAC International*, **101**(4), pp.1280–1282, DOI: 10.5740/jaoacint.SMPR2018.001.
 [21] AOAC (2016), *Guidelines for Standard Method Performance Requirements*, https://www.aoac.org/wp-content/uploads/2019/08/app_f.pdf, accessed 15 January 2021.
 [22] S.D. Seth, A. Beotra, S. Seth (1995), "Comparative bioavailability of two brands of norfloxacin", *Journal Association Physicians India*, **43**(5), pp.324–326.
 [23] D.N. Fish, A.T. Chow (1997), "The clinical pharmacokinetics of levofloxacin", *Clinical Pharmacokinetics*, **32**, pp.101–119, DOI: 10.2165/00003088-199732020-00002.
 [24] Y.H. Xu, D. Li, X.Y. Liu, et al. (2010), "High performance liquid chromatography assay with ultraviolet detection for moxifloxacin: Validation and application to a pharmacokinetic study in chinese volunteers", *Journal of Chromatography B*, **878**(32), pp.3437–3441, DOI: 10.1016/j.jchromb.2010.10.024.