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Original Research Article

Validation of HPLC-UV for simultaneous analysis of moxifloxacin and ciprofloxacin in peritoneal fluid

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Abstract

Purpose: *To develop and validate a novel HPLC-UV method that involves protein precipitation for quantifying ciprofloxacin and moxifloxacin in peritoneal fluid obtained from patients receiving treatment for continuous ambulatory peritoneal dialysis (CAPD).*

Methods: *Ice-cold (0.1 %) trifluoroacetic acid in methanol (v/v) was used to precipitate proteins in the peritoneal fluid samples. Chromatographic separation was achieved with the use of Agilent Zorbax SB-C18 analytical column (150 mm x 4.6 mm; 3.5 µm) under optimum chromatographic separation conditions (mobile phase: methanol – 0.1 % trifluoroacetic acid (34:66, v/v), flow rate of 1 mL/min, column temperature of 35°C, UV detection at 285 nm). Validation was done in accordance with the International Council for Harmonization (ICH) M10 guideline.*

Results: *Total run time was 13 min, validation process was linear (concentration range of 0.2 – 50 µg/mL) with correlation coefficients of 0.9987 and 0.9857 for ciprofloxacin and moxifloxacin, respectively. Relative recovery values and relative standard deviation (RSD) were acceptable. Based on ICH M10, precision and accuracy that were within-run and those that were between-run were good for the proposed method.*

Conclusion: *The HPLC-UV method developed and validated for quantifying ciprofloxacin and moxifloxacin found in the peritoneal fluid taken from patients undergoing CAPD is reliable. This method may be applicable for therapeutic drug monitoring, and conducting further pharmacokinetic studies on moxifloxacin and ciprofloxacin.*

Keywords: *Peritoneal fluid, Pharmacokinetics, Fluoroquinolones, Therapeutic drug monitoring, Validation, Moxifloxacin, Ciprofloxacin*

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INTRODUCTION

Moxifloxacin and ciprofloxacin are considered broad-spectrum antibiotics with a fluoroquinolone structure. Ciprofloxacin has proven to be highly effective in combating *Pseudomonas* species. The effect is also great with strains of *Enterobacteriaceae* and *Staphylococcus aureus*,

which are resistant to gentamicin and methicillin respectively. As a result, bone, soft tissue, and skin infections, as well as gastrointestinal, respiratory and urinary infections, are treated with ciprofloxacin [1]. Also, moxifloxacin is efficacious when administered to counter Gram-positive bacteria, as well as atypical and anaerobic pathogens.

It is possible to treat both resistant tuberculosis and pneumonia by utilizing ciprofloxacin and moxifloxacin [1,2]. Fluoroquinolones exhibit concentration-dependent bactericidal activity. Thus, to optimize the therapeutic effect and avoid antimicrobial resistance, it is imperative to determine their concentration in body fluids. Furthermore, emphasis on the dosage of the antibiotic and potential antimicrobial resistance have to be considered [3]. Therefore, this study validated high-performance liquid chromatography for UV-detection (HPLC-UV) following ICH M10 guidelines [4] and Q2 (R1) guidelines on Analytical Process Validation [5]. This is to enable quick determination of concentrations of moxifloxacin and ciprofloxacin in the peritoneal fluid. This method may be used for optimizing dosages of ciprofloxacin and moxifloxacin in peritonitis treatment.

EXPERIMENTAL

Drugs and materials

Ciprofloxacin (Figure 1 A) and moxifloxacin (Figure 1 B) were obtained from Hemofarm, Vršac, Serbia. Methylparaben (Figure 1 C; internal standard) was received in the form of a standard solid compound from Sigma-Aldrich, Germany, methanol (gradient grade) for liquid chromatography was provided by Avantor Performance Materials (Deventer, Netherlands), trifluoroacetic acid required for HPLC was obtained from Fisher Chemical, UK. High-performance liquid chromatography (HPLC; 1200 Agilent Technologies, USA), 1200 Series High-Performance Autosampler G1367B (Agilent Technologies, USA), HPLC-grade water (Smart 2 Pure purification system (TKA, Niederelbert, Germany), regenerated 0.45 µm cellulose membrane filters (Agilent, Germany).

Chromatographic conditions

A methanol (0.1 %) trifluoroacetic acid (34: 66 v/v) mixture constituted the mobile phase. The temperature of the column was 35 °C, flow rate was 1 mL/min. Furthermore, analytical column Zorbax SB-C18 (particle size: 3.5 µm, 150 mm x 4.6 mm i.d; Agilent Technologies, USA) separated the compounds. Data was collected using Microsoft Excel and the Agilent's ChemStation software was used for statistical processing. UV detection was conducted at 285 nm.

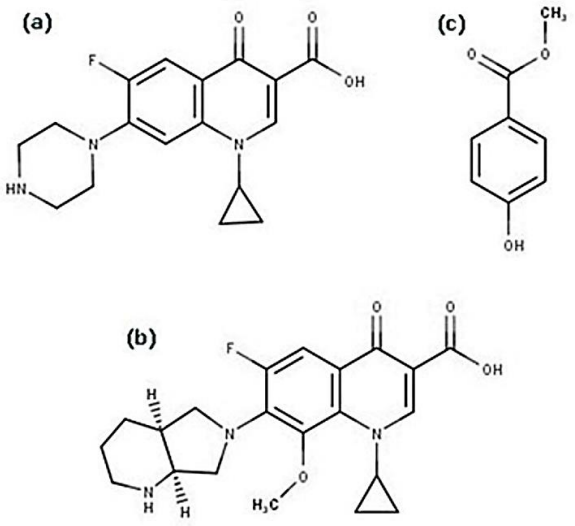

Figure 1: Structural forms of ciprofloxacin (a), moxifloxacin (b) and methylparaben (c)

Peritoneal fluid samples

Approval was obtained from the Ethics Committee of the University of Niš, Faculty of Medicine, Serbia (approval no. 12-519/3). The study was conducted in accordance with the Declaration of Helsinki [6], and the patients had given their consent in writing, stating that they had been informed. Peritoneal fluids were taken from CAPD treatment patients who were not administrated ciprofloxacin and moxifloxacin [7]. The fluids were stored at -20 °C prior to use. Only patients on CAPD are able to provide blank (drug-free) peritoneal fluid samples.

Standard solutions, quality control samples and calibration standards

Standard ciprofloxacin and moxifloxacin stock solution (5 mg/mL) were prepared with water. The solutions were freshly made daily. Preparation of standard stock solution which contained internal standard methylparaben was prepared in methanol (5 mg/mL) and stored at -20 °C. Internal standard solution of 1 mg/mL was prepared in methanol and stored at 4 - 8 °C for a month. Calibration standards were made by using peritoneal fluid taken from CAPD treatment patients who did not receive ciprofloxacin or moxifloxacin. Seven Eppendorf tubes were filled to 300 µL with the peritoneal fluid. Thereafter, 0.06, 0.18, 1.5, 4.5, 7.5, 13.5 and 15 µL of the standard 1 mg/mL solutions of moxifloxacin and

ciprofloxacin were transferred into the tubes. Every Eppendorf tube was also filled with 6 µL of standard 1 mg/mL methylparaben. Ciprofloxacin and moxifloxacin concentrations were 0.2, 0.6, 5, 15, 25, 45 and 50 µg/mL. Furthermore, the concentration of methylparaben was 20 µg/mL in each calibration standard.

Preparation of control samples involved adding the internal standard to the peritoneal fluid (without any ciprofloxacin and moxifloxacin) at a concentration of 20 µg/mL. Preparation of peritoneal fluid samples for quality control (QC) was done in the final moxifloxacin and ciprofloxacin concentrations, which were 0.6 µg/mL for low QC, 25 µg/mL for medium QC and 45 µg/mL for high QC. Similarly, 20 µg/mL was the internal standard concentration. The process of optimizing the procedure for sample preparation was achieved with the QC (medium) samples. All QC samples were prepared daily. Portions of all the QC samples (the high and low ones) were kept at -20 °C for testing stability once the freeze-thaw cycles have been completed, and long-term stability.

Procedure for preparing samples

A 300 µL peritoneal fluid sample aliquot (with no moxifloxacin or ciprofloxacin) was transferred into an Eppendorf tube (1 mL), after which 6 µL of the 1 mg/mL internal standard in methanol and 594 µL of 0.1 % trifluoroacetic acid in methanol (v/v). All the tubes were covered with a cap, vortexed, mixed for 5 min, and then frozen for 5 min at -20°C. The tubes were centrifuged for 10 min at 14.000 rpm and 4 °C. Thereafter, the HPLC apparatus was filled with the supernatant for analysis purposes at 25 °C.

Accuracy and precision

A different stock solution was used for preparing the quality control samples (QCs) and the calibration standards so that there would be no biased estimations that were unrelated to the analytical performance of the method. While the method validation was in progress, four concentration levels of the QCs for accuracy and precision runs were prepared within the calibration curve range (concentrations of high QC (45 µg/mL), medium QC (25 µg/mL), low QC (0.6 µg/mL) and LLOQ (0.2 µg/mL) of the peritoneal samples for MOX and CIP. The QCs within each run and those in different runs were analyzed to determine precision and accuracy. More specifically, six replicates were analyzed to assess within-run accuracy and precision at each concentration level of QC for every analytical run. Between-run precision and accuracy were assessed by analyzing every QC concentration level at six analytical runs for three consecutive days.

Method validation

Method validation was performed following ICH guideline M10 on validation of bioanalytical methods [4] and ICH Q2(R1) guidelines on analytical procedure validation [5]. Validation characteristics such as specificity, selectivity, matrix effect, precision and accuracy, calibration curve and range, carry-over, dilution integrity and stability were evaluated.

RESULTS

Selectivity

There was no observable response with the blank samples which may have been attributed to interfering components.

Specificity

The method's specificity was proven because there was no observable co-elution at the different retention times of ciprofloxacin, moxifloxacin and the internal standard from the freshly prepared and spiked peritoneal fluid samples at 50 µg/mL (the upper limit of quantification, i.e. ULOQ) and 0.2 µg/mL (the lower limit of quantification, i.e. LLOQ) for ciprofloxacin and moxifloxacin compared to the peritoneal fluid samples with no moxifloxacin and ciprofloxacin (Figure 2).

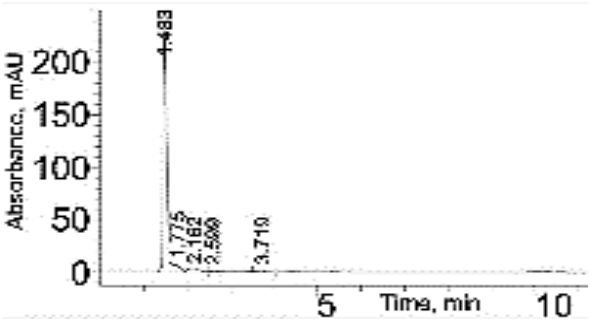

Figure 2: The chromatogram which is representative of the peritoneal fluid sample without any CIP and MOX

Matrix effect

Three high QC and low QC replicates were used for evaluating the matrix effect, each prepared by using the peritoneal fluid samples of six CAPD treatment patients who were not given ciprofloxacin or moxifloxacin. Accuracy for each patient, ranged between 85 and 115 % of the

nominal concentration, while the precision did not exceed 15 % (Table 1).

Calibration curve and range

After seven calibration standards in triplicate on three consecutive days, the ciprofloxacin and moxifloxacin calibration curves were generated. The two calibration curves were prepared based on Eq 1 and Eq 2 at 0.2 - 50 µg/mL.

$y = 0.6044x - 0.3053$; $r^2 = 0.9987$ for ciprofloxacin ……. (1)
$y = 0.3610x - 0.3034$; $r^2 = 0.9857$ for moxifloxacin ……. (2)

Where y is the ratio of the peak area, x is concentration, and r is the correlation coefficient. The confidence factor (t_α), standard slope deviation (Sa), and standard intercept deviation (Sb) are presented below:

Sa = 0.1383, Sb = 3.8419 and t_α = 0.0795 for ciprofloxacin and Sa = 0.0761, Sb = 2.1141 and t_α = 0.1435 for moxifloxacin.

Tabular value of t_α (p = 0.05 and t_{tab}= 2.37) was compared with the values of t_α calculated for ciprofloxacin and moxifloxacin. For this reason, the intercepts' deviation from the zero value was not significant.

Chromatogram of peritoneal samples

Figure 3 shows a representative of the peritoneal fluid sample following spiking with 20 µg/mL of the internal standard (IS). Furthermore, Figure 4 shows the chromatogram of the representative peritoneal fluid sample which was spiked with MOX and CIP of 25 µg/mL and the IS of 20 µg/mL.

Back-calculated concentrations

The calculated mean precision and accuracy values were presented alongside the back-calculated concentrations for seven calibration standards. All the standards for calibration at the seven levels of concentration varied between 85 – 115 % of the nominal concentration and 80 – 120 % for the lower limit of quantification (LLOQ) level (Table 2). The limit of detection (LOD) was 0.02 µg/mL for CIP and MOX. The lower limit of quantification (LLOQ) was 0.2 µg/mL for both analytes.

Accuracy and precision

At each concentration level, accuracy of the nominal concentration ranged from 85 to 115 %, with the exception of LLOQ, whose nominal concentration ranged from 80 to 120 %. The precision value did not exceed 15 % at each concentration level except for LLOQ. An analysis was also made for high QC, medium QC and low QC levels of concentration for non-accuracy and precision validation runs, in duplicate (Table 3 and Table 4).

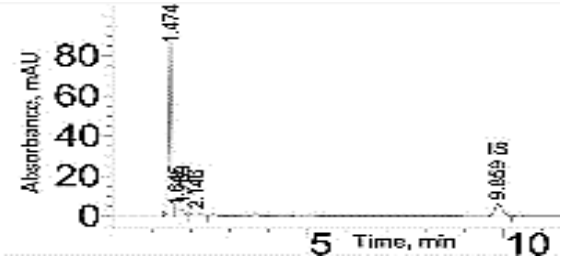

Figure 3: Representative chromatogram of the peritoneal fluid sample following spiking with IS at 20 µg/mL

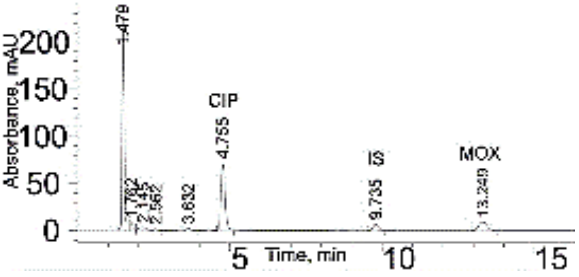

Figure 4: Representative chromatogram of the peritoneal fluid sample following spiking with MOX and CIP at 25 µg/mL and IS of 20 µg/mL

Table 1: Matrix effect evaluation at low QC (0.6 µg/mL) and high QC (45 µg/mL) in peritoneal fluid samples from six patients for CIP and MOX (n = 3)

Analyte	Parameter	Concentration of CIP and MOX, µg/mL	
		0.6	***45***
CIP	Precision (RSD / %)	13.84	9.49
	Accuracy (R / %)	89.25	112.44
	Determined conc., µg/mL	0.53	50.60
MOX	Precision (RSD / %)	8.98	12.69
	Accuracy (R / %)	94.42	106.87
	Determined conc., µg/mL	0.57	48.09

Table 2: Back-calculated concentrations of 7 standards of calibration with calculated mean accuracy and precision values for CIP and MOX (n = 3)

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX						
		0.2	***0.6***	***5***	***15***	***25***	***45***	***50***
CIP	Precision RSD (%)	16.35	12.79	11.34	7.22	10.68	13.37	12.83
	Accuracy, R (%)	87.57	92.48	112.74	92.62	108.29	106.32	89.72
	Det. Conc (µg/mL)	0.17	0.55	5.64	13.89	27.11	47.84	44.86
MOX	Precision, RSD (%)	15.97	14.02	8.76	11.57	13.91	12.96	12.58
	Accuracy, R (%)	110.84	112.28	90.86	108.51	94.33	96.72	107.35
	Det. Conc (µg/mL)	0.22	0.67	4.54	16.28	23.58	43.52	53.67

Table 3: Evaluation of precision and accuracy (within-run) at LLOQ concentrations (0.2 µg/mL), low QC (0.6 µg/mL), medium QC (25 µg/mL) and high QC (45 µg/mL) of peritoneal samples for CIP and MOX (n = 6)

Analyte	Parameter	Concentration of CIP and MOX, µg/mL			
		0.2	***0.6***	***25***	***45***
CIP	Recovery (%)	11.84	12.07	8.59	12.47
	RSD (%)	109.74	93.27	107.21	104.38
	Determined conc (µg/mL)	0.22	0.56	26.80	46.97
MOX	Recovery (%)	13.94	14.46	9.82	10.42
	RSD (%)	112.51	90.15	110.21	93.81
	Determined conc (µg/mL)	0.22	0.54	27.55	42.21

Table 4: The evaluation of the precision and accuracy (between-run) at the concentrations of LLOQ (0.2 µg/mL), low QC (0.6 µg/mL), medium QC (25 µg/mL) and high QC (45 µg/mL) in the samples of peritoneal fluid for CIP and MOX (*n* = 6)

Analyte	Parameter	Concentration of CIP and MOX, µg/mL			
		0.2	***0.6***	***25***	***45***
CIP	Recovery (%)	16.85	13.92	11.54	14.47
	RSD (%)	83.51	114.58	112.72	89.59
	Determined conc (µg/mL)	0.17	0.69	28.18	40.31
MOX	Recovery (%)	18.16	14.94	12.82	10.42
	RSD (%)	115.74	113.28	89.43	91.36
	Determined conc (µg/mL)	0.23	0.68	22.35	41.11

Table 5: Non-accuracy and precision validation runs at the concentrations of low QC (0.6 µg/mL), medium QC (25 µg/mL) and high QC (45 µg/mL) in the samples of peritoneal fluid for CIP and MOX (n = 2)

Analyte	Parameter	Concentration of CIP and MOX, µg/mL		
		0.6	***25***	***45***
CIP	Recovery (%)	10.73	9.67	11.57
	RSD (%)	89.76	108.53	105.38
	Determined conc (µg/mL)	0.54	27.13	47.42
MOX	Recovery (%)	8.62	12.23	10.25
	RSD (%)	107.41	93.49	103.76
	Determined conc (µg/mL)	0.64	23.37	46.69

Carry-over

Assessment of carry-over was undertaken through an analysis of six blank samples after the calibration standard at 50 µg/mL (ULOQ). While each blank sample was analyzed, there were no observable peaks at the times of the retention of MOX, CIP and the IS. Hence, no carry-over was present at that time.

Dilution integrity

Dilution QCs were made using analyte concentrations in the matrix that were higher than those of ULOQ and thereafter diluted with the blank matrix. Following this, tests were conducted on six replicates per dilution factor in a single run to determine whether the concentrations had been measured precisely and accurately within the calibration range.

Concentrations and dilution factors, while the study sample analysis was in progress, were all within the range of the concentrations and dilution factors assessed at the time of validation. The QC mean dilution accuracy was 85-115 % of the nominal concentration, whereas the precision did not exceed 15 % (Table 6).

Stability

At every QC level, the mean concentration was 85 – 115 % of the nominal one. The freeze-thaw stability in the matrix –i.e. how stable the analyte was upon completion of three freezing and thawing cycles was also examined. High QC and low QC remained frozen for a period of 12 h between the thawing cycles (Table 7).

Short-term stability

Short-term stability within the matrix–high QC and low QC were thawed at 25 °C for 8 h (Table 8).

Table 6: Dilution integrity for dilution QCs at 100 µg/mL, 150 µg/mL,200 µg/mL and 250 µg/mL (n = 6)

Dilution QC concentration (µg/mL)		**100**	**150**	**200**	**250**
Dilution factor expressed as a ratio		1/20	1/10	1/8	1/5
Concentration (µg/mL) of CIP and MOX		5	15	25	50
Analyte	***Parameter***				
CIP	Precision, RSD (%)	12.89	10.85	9.31	14.21
	Accuracy, R (%)	92.49	112.87	87.39	110.25
	Det. Conc (µg/mL)	4.62	16.93	21.85	55.12
MOX	Precision, RSD (%)	13.64	9.98	12.35	11.64
	Accuracy, R (%)	113.75	107.92	94.19	89.82
	Det. conc (µg/mL)	5.69	16.19	23.54	44.91

Table 7: Freeze-thaw stability at low QC and high QC in peritoneal samples (n = 6)

Analyte	**Parameter**	**Concentration of CIP and MOX (µg/mL)**	
		0.6	***45***
CIP	Recovery (%)	89.24	93.26
	RSD (%)	8.91	11.51
	Determined conc (µg/mL)	0.53	41.97
MOX	Recovery (%)	112.76	106.71
	RSD (%)	12.75	10.83
	Determined conc (µg/mL)	0.68	48.02

Table 8: Short-term stability at low QC and high QC in peritoneal fluid samples (n = 6)

Analyte	**Parameter**	**Concentration of CIP and MOX (µg/mL)**	
		0.6	***45***
CIP	Recovery (%)	94.28	108.03
	RSD (%)	9.71	12.69
	Determined conc (µg/mL)	0.56	48.61
MOX	Recovery (%)	111.98	91.83
	RSD (%)	11.51	10.36
	Determined conc (µg/mL)	0.67	41.32

Long-term stability

Long-term stability within the matrix – high QC and low QC were kept in the freezer at -20 °C for 3 months (Table 9).

Stability of analyte

Stability data for the processed samples at 4 °C for 24 h are listed in Table 10.

Stability of analyte and IS in working solutions

The stability of the analyte and the IS in working solutions are shown in Table 11 and Table 12.

DISCUSSION

Taking into consideration the contemporary literature to date, the determination of ciprofloxacin and moxifloxacin through the use of HPLC methods have been conducted in human serum [8,9], plasma [10,11], peritoneal exudate [12], urine [11,13], amniotic fluid [14], ascites [15], or peritoneal fluid [16-17]. Such studies made use of UV detection [6,10,11,14,17], fluorescence detection [11,16] and MS detection [8].

Table 9: Long-term stability at low QC and high QC in peritoneal fluid samples (n = 6)

Analyte	Parameter	Concentration of CIP and MOX (µg/mL)	
		0.6	***45***
CIP	Recovery (%)	108.84	89.53
	RSD (%)	10.47	13.28
	Determined conc (µg/mL)	0.65	40.28
MOX	Recovery (%)	105.34	91.58
	RSD (%)	12.94	11.86
	Determined conc (µg/mL)	0.63	41.21

Table 10: Stability of the analyte at low QC and high QC (n = 6)

Analyte	Parameter	Concentration of CIP and MOX (µg/mL)	
		0.6	***45***
CIP	Recovery (%)	107.85	109.17
	RSD (%)	9.53	10.82
	Determined conc (µg/mL)	0.65	49.13
MOX	Recovery (%)	110.35	94.57
	RSD (%)	12.59	8.58
	Determined conc (µg/mL)	0.66	42.56

Table 11: Stability of the analyte and the IS in stock solutions (n = 6)

Analyte	**CIP**			**MOX**			**IS**		
Conc (mg/mL)		***5***			***5***			***5***	
Dilution factor expressed as a ratio	1/1000	1/200	1/100	1/1000	1/200	1/100	1/1000	1/250	1/100
Nominal concentration (µg/mL)	5	25	50	5	25	50	5	20	50
Determined Concentration (µg/mL)	4.90	25.48	49.63	5.05	24.90	49.78	5.08	19.68	50.38

Table 12: Stability of the analyte and IS in working solutions (n = 6)

Analyte	**CIP**			**MOX**			**IS**		
Conc (mg/mL)		**1**			**1**			**1**	
Dilution factor expressed as a ratio	1/200	1/40	1/20	1/200	1/40	1/20	1/200	1/50	1/20
Nominal conc (µg/mL)	5	25	50	5	25	50	5	20	50
Determined Concentration (µg/mL)	5.09	25.23	50.47	4.92	25.48	50.85	4.97	20.31	49.58

The methods used for performing sample pre-treatment were protein precipitation [8,6,15], liquid extraction [16], continuous dialysis [9], filtration using a membrane filter (0.45 µm) before dilution is performed [17] or has been completed [11]. The method proposed in this study was protein precipitation for peritoneal fluid sample purification and protein removal. Once this was completed, the supernatant was injected into the HPLC system. The evaporation step and reconstitution were avoided to simplify the sample pre-treatment. Sample preparation procedure involves using a small peritoneal fluid volume, which is highly suitable for the manipulation of samples while the method is being developed and validated. Furthermore, the sample pre-treatment time was around 20 min, whereas chromatographic run lasted for approximately 13 min, convenient for applying the described method in practice.

Previously published methods do not have as many advantages as those introduced by the proposed method of HPLC-UV to establish the presence of ciprofloxacin and moxifloxacin in peritoneal fluid samples. Unlike the HPLC-MS apparatus, HPLC-UV apparatus is not expensive, which signifies a crucial advantage for application in clinical practice. The proposed method is employed for simultaneously determining the presence of ciprofloxacin and moxifloxacin in peritoneal fluid, which may be considered to be an improvement compared to previously published ciprofloxacin [16] or moxifloxacin [17] determination methods. The concentration range of the HPLC-UV method for the two analytes was 0.2 - 50 µg/mL, which is wider compared to previous studies [16] or moxifloxacin [17] analysis method. Furthermore, the method suggested may be applicable not only for monitoring drugs used in therapy but also for studying the pharmacokinetics of moxifloxacin and ciprofloxacin with improved sensitivity.

There was no observable response of any interfering components at CIP, MOX and IS retention times. Specificity of the method was proven because there was no observable co-elution at the retention times of ciprofloxacin, moxifloxacin and IS from the freshly made spiked samples of the peritoneal fluid at 50 and 0.2 µg/mL compared to peritoneal fluid samples with no moxifloxacin and ciprofloxacin. Following evaluation of the matrix effect, there was no observable analyte response alteration owing to any interfering components in the sample matrix. Also, R^2 value for ciprofloxacin and moxifloxacin at 0.2 – 50 µg/mL was 0.9987 and 0.9857, respectively, suggesting good linearity pattern. Furthermore, the student's t-test indicated that the intercepts' deviation from zero was not significant. Relative recovery (R in %) values for the back-calculated concentration of each standard used for calibration were cited in ICH M10 guidelines [4]. All the calibration standards met ICH M10 criteria at seven concentration levels. Furthermore, considering the fact that the analysis was conducted in triplicate, all the standards of calibration for seven levels of concentration were in line with the same criteria per level of concentration.

Values of relative recovery (R in %) and relative standard deviation (RSD in %) were within acceptable range under ICH M10 guidelines [4]. As a result, the within-run and between-run precision and accuracy of this method were good. The blank samples were analyzed after completion of the calibration standard at ULOQ, and no peaks were observed at retention time of MOX, CIP and IS. Thus, there was no carry-over. The dilution integrity assessment has proven that the dilution procedure used for the samples does not have an impact on the precision and accuracy of MOX and CIP concentrations. Also, both MOX and CIP were stable in all the investigations conducted.

CONCLUSION

The HPLC-UV method developed and validated in this study is dependable and efficiently analyses the concentration of ciprofloxacin and moxifloxacin in peritoneal fluid samples taken from patients on CAPD. Thus, this approach may be employed to monitor peritoneal drug levels and in pharmacokinetic studies.

DECLARATIONS

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Ethical approval

Ethical approval was obtained from the Ethics Committee of the Faculty of Medicine, University of Niš, Serbia (approval no. 12-519/3).

Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We hereby declare that this article was prepared by all the authors cited herein and that all the liabilities pertaining to the claims relating to the content hereof will be borne by the authors. Associate Professor Predrag Džodić, PhD, conceived and designed the study, collected data and wrote the manuscript.

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