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Original Research Article

A reliable chromatographic method for the simultaneous determination of ciprofloxacin and moxifloxacin in human serum

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Abstract

Purpose: *To develop and validate a simple chromatographic method for the analysis of ciprofloxacin and moxifloxacin in human serum.*
Methods: *After protein precipitation had been performed, high performance liquid chromatography (HPLC) with UV detection was utilized for the analysis of ciprofloxacin and moxifloxacin in human serum. Analytical column Zorbax SB-C18 (150 mm x 4.6 mm i.d., particle size 3.5 µm) was used as a stationary phase. Chromatographic separation was realized with the mobile phase 0.1% trifluoroacetic acid in water for chromatography - methanol (66:34, v/v), at the flow rate of 1 mL/min, temperature of 35 °C and detection at 280 nm. The method validation was performed according to the guidelines of the European Medicines Agency (EMA).*
Results: *The chromatographic run time was about 12 minutes and no interference was observed. For ciprofloxacin, the method was linear over a concentration range of 0.5-50 µg/mL, with a correlation coefficient of 0.9874. For moxifloxacin, the method was linear over a concentration range of 0.5-50 µg/mL, with a correlation coefficient of 0.9946. Since relative standard deviation (RSD) and relative recovery values were within acceptable limits according to EMA guidelines, good intra-day precision, inter-day precision, as well as the accuracy of the method, were observed.*
Conclusion: *A simple and reliable HPLC-UV method has been developed and validated for the simultaneous determination of ciprofloxacin and moxifloxacin in human serum. The method can be applied for therapeutic drug monitoring but also and pharmacokinetic studies of ciprofloxacin and moxifloxacin.*

Keywords: *Human serum, Ciprofloxacin, Moxifloxacin, Protein precipitation, HPLC, UV detection, Method validation*

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INTRODUCTION

Ciprofloxacin (CIP) and moxifloxacin (MOX) belong to fluoroquinolones, a class of broad-spectrum antibiotics affecting both Gram-positive and Gram-negative bacteria [1]. CIP is a fluoroquinolone with notable antimicrobial activity against *Pseudomonas* species, *Enterobacteriaceae* strains resistant to gentamicin, as well as methicillin-resistant *Staphylococcus aureus* strains. It is usually used for the treatment of urinary, respiratory and gastrointestinal tract infections, some skin, bone, and soft tissue infections [2,3].

Moxifloxacin (MOX), a fourth-generation fluoroquinolone, has a prolonged effect on Gram-positive pathogens, including *Streptococcus pneumoniae*, atypical organisms and anaerobes. This so-called, "respiratory quinolone" is also used in the eradication of *Helicobacter pylori*. CIP and MOX are both recommended in the treatment of community-acquired pneumonia and as second-line agents for multidrug-resistant tuberculosis [2,4-6].

Owing to worrying antimicrobial resistance, the optimization of traditional antibiotic dosing strategies has received a lot of attention in recent years [7-9]. Fluoroquinolones exhibit concentration-dependent bactericidal activity, so it is important to monitor concentration levels during therapy to accomplish an optimal effect and to avoid resistance [9-11].

Therefore, the target of the present study is the development and validation of a cost-effective method for realizing a simple and reliable high-performance liquid chromatography with UV detection (HPLC-UV) for determining CIP and MOX simultaneously in human serum, which could easily be applied to the therapeutic drug monitoring of CIP and MOX.

Validation was performed according to European Medicines Agency (EMA) guidelines [12] and International Conference on Harmonisation (ICH) guidelines [13]. The proposed method was fully validated in terms of selectivity, linearity, sensitivity, accuracy, precision, absolute recovery and stability of the analytes.

EXPERIMENTAL

Drugs and materials

Standard compounds CIP and MOX, in solid form, were kindly donated by the pharmaceutical company Hemofarm (Vršac, Serbia). Internal standard methylparaben (IS), as solid standard compound, was purchased from Sigma-Aldrich (Steinheim, Germany). Methanol gradient grade for liquid chromatography was purchased from Avantor Performance Materials (Deventer, The Netherlands) [14]. Trifluoroacetic acid (TFA) for high-performance liquid chromatography (HPLC) was purchased from Fisher Chemical (Loughborough, UK).

Apparatus

HPLC analysis was performed using the chromatographic system Agilent Technologies 1200 (Wilmington, DE, USA). A sample of 10 µL was injected with Agilent 1200 Series High Performance Autosampler G1367B. HPLC-grade water was acquired from the purification system Smart 2 Pure (TKA, Niederelbert, Germany). The mobile phase was de-gassed and purified by vacuum filtration through 0.45 µm regenerated cellulose membrane filters (Agilent, Böblingen, Germany). Zorbax SB-C18 analytical column (150 mm x 4.6 mm i.d., particle size 3.5 µm) (Agilent, USA) was used for compounds separation [14]. Data were collected using Agilent's ChemStation software. The Microsoft Excel software was used for statistical processing [14].

Chromatographic conditions

The mobile phase was a mixture of 0.1% trifluoroacetic acid in water-methanol (66:34, v/v). The flow rate was set at 1 mL/min and the column temperature at 35°C. Ultraviolet (UV) detection was performed at 280 nm.

Human serum samples

Ethical approval for this work was obtained from the Ethics Committee (Ethics Committee of Faculty of Medicine, University of Niš, Serbia; approval number 12-519/3) [14]. The investigations followed the international guidelines, WMA Declaration of Helsinki [15]. Healthy volunteers and patients gave their written informed consent. Blank serum was obtained from ten different healthy volunteers (Clinical Centre Niš, Niš, Serbia). Whole blood was taken by means of venipuncture into vacutainers and, after coagulation, separated by centrifugation at 3000 g for 15 min [14]. All serum samples were stored at -20°C until analysis [14].

Standard solutions, calibration standards and quality control samples

Standard stock solution and standard working solution of CIP contained 5 mg/mL and 1 mg/mL in water, respectively. Standard solutions of MOX

were prepared in the same way. All standard stock and working solutions of CIP and MOX were prepared fresh every day [14].

A standard stock solution of IS was prepared in methanol at a concentration of 5 mg/mL and kept at -20°C. A standard working solution of IS of 1 mg/mL in methanol was made and stored at 4-8°C for 1 month.

For calibration standards, 300 µL of blank human serum was transferred into each of the seven Eppendorf tubes. Afterwards, each of the seven tubes received one of the following volumes 0.15, 0.45, 1.5, 4.5, 7.5, 13.5 and 15 µL of 1 mg/mL CIP and MOX standard working solutions. A volume of 6 µL of the 1 mg/mL IS standard working solution was added to each of the Eppendorf tubes. The concentrations of CIP and MOX in calibration standards were 0.5, 1.5, 5, 15, 25, 45 and 50 µg/mL. Moreover, the concentration of IS in each calibration standard was 20 µg/mL.

Zero plasma samples were prepared by adding IS to the blank serum (serum without drugs) in the concentration of 20 µg/mL [14].

Quality control (QC) serum samples were prepared ~~to~~ in the final concentrations of 1.5 µg/mL (low QC), 25 µg/mL (medium QC) and 45 µg/mL (high QC) of CIP and MOX. The concentration of IS was 20 µg/mL. For the optimization of sample preparation procedure, QC (medium) samples were used.

Low QC, medium QC and high QC samples were prepared fresh every day. In order to test stability after freeze-thaw cycles as well as long term stability, parts of all low QC and high QC samples were kept at -20°C [14].

Sample preparation procedure

A 300 µL aliquot of blank human serum was transferred to a 1.0 mL Eppendorf tube, followed by 6 µL of 1 mg/mL IS in methanol and 594 µL of 0.1% TFA in methanol (v/v). Each tube was capped, vortexed and mixed for 5 min, and frozen for 15 min at -20°C. After thawing, each tube was centrifuged for 10 min at 10000 rpm and 4°C. Finally, at the temperature of 25°C, the supernatant was injected into the HPLC apparatus for analysis [14].

Validation

Validation of the proposed method was performed according to European Medicines Agency (EMA) guidelines for Bioanalytical method validation [12], as well as the International Conference on Harmonisation (ICH) guidelines [13]. The following validation characteristics were considered: selectivity, linearity, sensitivity, accuracy, precision, absolute recovery and stability of the analytes [14].

RESULTS

Selectivity

To test the selectivity of the method, freshly prepared spiked serum samples of 50 µg/mL, as well as 0.5 µg/mL (lower limit of quantification level, LLOQ) of CIP and MOX, were compared to blank serum (without drugs) obtained from ten healthy volunteers. Co-elution was not observed at the retention times of CIP, MOX and IS [14]. Figure 1 shows the representative chromatogram of blank serum.

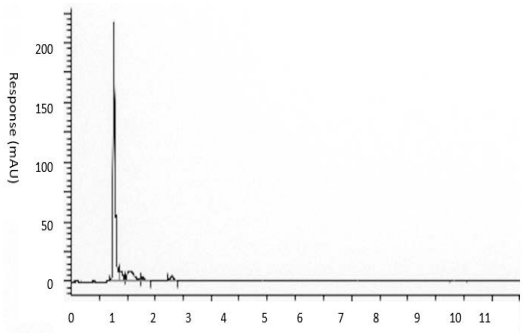

Figure 1: Representative chromatogram of blank serum

In order to thoroughly investigate the selectivity, serum samples were assayed from volunteer patients who were administrated the following drugs: alfacalcidol, amlodipine, atorvastatine, bisoprolol, bromazepam, cefixime, digoxin, felodipine, fluconazole, folic acid, fosinopril, furosemide, irbesartan, isosorbide dinitrate, lercanidipine, metronidazole, nebivolol, pantoprazole, ramipril, rosuvastatin, spironolactone, tamsulosin, telmisartan and warfarin. Co-elution was not observed at the retention times of CIP, MOX and IS [14].

Linearity

The calibration curves for CIP and MOX were generated after the analysis of seven calibration standards. For the examined concentration range for CIP and MOX (0.5-50 µg/mL), the two calibration curves were obtained based on Eqs 1 and 2.

$y = 0.1901x + 0.0417$; $r^2 = 0.9874$ for CIP ……. (1)

$y = 0.0930x + 0.0303$; $r^2 = 0.9946$ for MOX ……. (2)

where y is the peak area ratio, x is the concentration of the compound, and r is the correlation coefficient [16]. The intercepts of the calibration curves were tested using Student's t-test [16]. The following results were calculated for the standard deviation of the slope (Sa), standard deviation of the intercept (Sb) and the confidence factor (t_α) [16]:

Sa = 0.0691, Sb = 1.9199 and t_α= 0.0217 for CIP; and Sa = 0.0041, Sb = 0.1151 and t_α= 0.2635 for MOX. The calculated t_α values for CIP and MOX were compared to the tabular t_α value (p = 0.05 and t_{tab} = 2.37), and thus the deviation of the intercepts from the zero value was insignificant [14, 16]. Figure 2 shows the representative chromatogram of blank serum spiked with IS. Figure 3 shows the representative chromatogram of blank serum spiked with CIP, MOX and IS.

Limit of detection (LOD) and lower limit of quantification (LLOQ) values for the two analytes were 0.05 and 0.5 µg/mL, respectively. The accuracy and precision were evaluated in six replications (n = 6) at the LLOQ level. Accuracy is reported as recovery (R in %) and precision is reported as relative standard deviation (RSD in %) [14]. The results obtained for LLOQ are presented in Table 1.

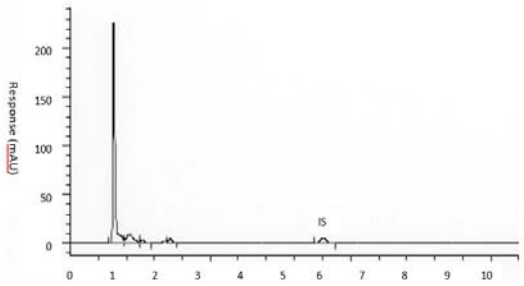

Figure 2: Representative chromatogram of blank serum spiked with 20 µg/mL of IS

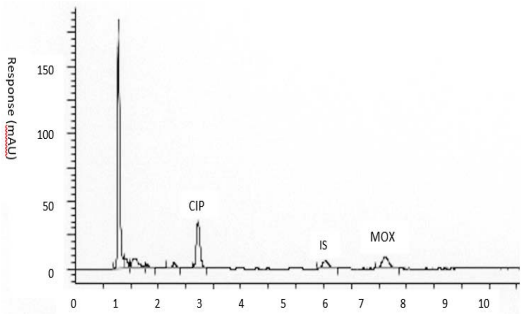

Figure 3: Representative chromatogram of blank serum spiked with 25 µg/mL of CIP and MOX, and 20 µg/mL of IS

Accuracy and precision

Intra-day and inter-day accuracy and precision (n = 6) was examined at low QC (1.5 µg/mL), medium QC (25 µg/mL) and high QC (45 µg/mL) levels. Accuracy is reported as recovery (R in %) and precision as relative standard deviation (RSD in %) [14].

Table 1: Intra-day precision and accuracy at LLOQ (0.5µg/mL), low QC (1.5 µg/mL), medium QC (25 µg/mL), and high QC (45 µg/mL) concentrations in serum samples for CIP and MOX (n = 6)

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX			
		0.5	***1.5***	***25***	***45***
CIP	Precision (RSD, %)	6.88	8.98	7.07	8.11
	Accuracy (R, %)	107.19	109.58	91.26	113.27
	Determined conc. (µg/mL)	0.54	1.64	22.81	50.97
MOX	Precision (RSD, %)	14.64	13.01	12.38	6.94
	Accuracy (R, %)	86.14	91.03	93.33	92.3
	Determined conc. (µg/mL)	0.43	1.37	23.33	41.53

Table 2: Inter-day precision and accuracy at low QC (1.5 µg/mL), medium QC (25 µg/mL), and high QC (45 µg/mL) concentrations in serum samples for CIP and MOX (n = 6)

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX		
		1.5	***25***	***45***
CIP	Precision (RSD, %)	7.62	6.01	10.07
	Accuracy (R, %)	113.75	109.91	97.22
	Determined conc. (µg/mL)	1.71	27.48	43.75
MOX	Precision (RSD, %)	11.55	10.68	5.23
	Accuracy (R, %)	94.14	88.92	109.29
	Determined conc. (µg/mL)	1.41	22.23	49.18

The results obtained for RSD (%) and recovery (R, %) are in accordance with the EMA [12] guidelines: at LLOQ level, a precision of 20% and accuracy of 80-120%; at low QC, medium QC and high QC levels, a precision of 15% and accuracy of 85-115%. The results obtained are presented in Table 1 and Table 2.

Absolute recovery

In order to evaluate the efficiency of the sample preparation procedure, absolute recovery values were calculated. Spiked drug-free serum samples at low QC, medium QC and high QC were subjected to the sample preparation procedure [14] (as explained in detail in the subsection on the Sample preparation procedure). The above-mentioned samples were compared to blank serum samples, that were extracted following the same sample preparation procedure, and subsequently spiked at the same concentration levels [14]. The absolute recovery values and the estimated concentrations from human serum for CIP and MOX are presented in Table 3.

Stability of the analytes

According to EMA guidelines [12], the following stability tests were performed at low QC and high QC levels: short-term stability, post-preparative stability, long-term stability and freeze-thaw cycles, and the results are presented in Table 4, Table 5, Table 6 and Table 7, respectively.

Table 3: Absolute recovery values for CIP and MOX from serum samples (n = 6)

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX		
		1.5	***25***	***45***
CIP	Recovery (%)	93.33	107.52	104.1
	RSD (%)	5.78	5.2	5.9
	Determined conc. (µg/mL)	1.4	26.88	46.84
MOX	Recovery (%)	88,97	113.32	92.7
	RSD (%)	8.22	8.87	6.38
	Determined conc. (µg/mL)	1.33	28.33	41.72

Table 4: Short-term stability results at low QC and high QC concentrations in serum samples (n = 6)

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX	
		1.5	***45***
CIP	Recovery (%)	88.54	105.14
	RSD (%)	11.59	7.35
	Determined conc. (µg/mL)	1.33	47.31
MOX	Recovery (%)	108.16	95.41
	RSD (%)	7.34	4.81
	Determined conc. (µg/mL)	1.62	42.93

Table 5: Post-preparative stability results at low QC and high QC concentrations in serum samples (n = 6)

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX	
		1.5	***45***
CIP	Recovery (%)	104.86	96.25
	RSD (%)	9.8	10.72
	Determined conc. (µg/mL)	1.57	43.31
MOX	Recovery (%)	111.26	92.52
	RSD (%)	13.71	7.27
	Determined conc. (µg/mL)	1.7	41.63

Table 6: Long-term stability results at low QC and high QC concentrations in serum samples (n = 6)

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX	
		1.5	***45***
CIP	Recovery (%)	108.33	94.65
	RSD (%)	6.93	4.93
	Determined conc. (µg/mL)	1.62	42.59
MOX	Recovery (%)	90.8	112.59
	RSD (%)	9.56	9.89
	Determined conc. (µg/mL)	1.36	50.66

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

Analyte	Parameter	Concentration (µg/mL) of CIP and MOX	
		1.5	***45***
CIP	Recovery (%)	110.42	105.98
	RSD (%)	9.43	6.06
	Determined conc. (µg/mL)	1.66	47.69
MOX	Recovery (%)	97.13	92.09
	RSD (%)	11.82	7.72
	Determined conc. (µg/mL)	1.46	41.44

DISCUSSION

According to literature data, the determination of CIP and MOX by HPLC methods has already been performed in human serum [7,17-23,30-34], plasma [3,4,10,24-29,35-40], urine [17,19,20], amniotic fluid [24], peritoneal fluid [18,19,35], peritoneal exudate [41] or ascites [31]. The detectors used in such published papers were: ultraviolet (UV) detection [3,4,17,21,24,26,29,30, 35,38,39], fluorescence detection [10,17-20,22,23,25-28,31,33,35-37,40,41] and MS detection [7,32,34]. Sample pretreatment was performed by protein precipitation [7,17,20-22,30,31,34], liquid extraction [18,32], continuous dialysis [23], or filtration through the 0.45 µm membrane filter, and subsequently dilution [19].

The method described in this paper involves protein precipitation for the purpose of removing proteins and purifying human serum samples. Subsequently, supernatant was injected into the HPLC system; thus, time-consuming evaporation and reconstitution steps were avoided.

Low human serum volume was used for sample preparation, which is convenient for sample handling during method development and validation. In addition, sample pretreatment lasted about 30 min and chromatographic run time was about 12 min, which is appropriate for the clinical application of the method. Moreover, CIP and MOX in human serum were determined simultaneously.

The proposed HPLC-UV method for the determination of CIP and MOX in human serum has some advantages over the previously published methods. The HPLC-UV apparatus is more affordable than the HPLC-MS one, which is of great importance for the method application in clinical practice. In addition, the method range is wider for the two analytes (0.5-50 µg/mL) in comparison with the published methods [4,7,12-14,17,24,27]. The proposed method showed good peak symmetry despite the previously observed peak tailing [21,33,34]. In addition, the method showed adequate sensitivity and could thus be applied to analysis of human serum samples in pharmacokinetic studies.

The proposed method is selective due to the fact that no co-elution was spotted at the retention times of CIP, MOX and IS from freshly prepared spiked samples at 50 µg/mL, as well as 0.5 µg/mL (LLOQ) for CIP and MOX, compared to blank plasma from ten healthy volunteers [14]. Moreover, an additional selectivity examination of the method was successfully performed with volunteer patients.

For the investigated range for CIP and MOX (0.5-50 µg/mL), r^2 was equal to 0.9874 and 0.9946, respectively. Therefore, the linearity of both calibration curves was proven to be good. Furthermore, according to student's t-test, the deviation of the intercepts from the zero value was insignificant [14,16].

The method showed good sensitivity for CIP and MOX since LOD and LLOQ values were 0.05 and 0.5 µg/mL, respectively.

Relative standard deviation (RSD in %) and relative recovery (R in %) values were within acceptable limits, according to EMA guidelines [12]. Therefore, a good intra-day precision, inter-day precision and accuracy of the method were observed.

Regarding the absolute recovery values of CIP and MOX, the sample preparation procedure showed an adequate efficiency. In addition, absolute recovery values appeared to be independent of concentration.

Considering the presented results, CIP and MOX stability was proven in all investigations.

CONCLUSION

A novel HPLC-UV method has successfully been developed and validated for the analysis of CIP and MOX in human serum. The method is simple, reliable and affordable. Furthermore, the proposed method can be applied to the analysis of CIP and MOX in human serum samples obtained from patients, not only for therapeutic drug monitoring, but also for pharmacokinetic studies.

DECLARATIONS

Acknowledgement

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Conflict of interest

The authors declare that no conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by all the authors stated below in this article, and that all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Assistant Professor Predrag Džodić, PhD conceived and designed the study, collected data and wrote the manuscript.

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