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Validation of a MLC method with fluorescence detection for the determination of quinolones in urine samples by direct injection

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ABSTRACT

A sensitive and robust method was developed and validated for the routine identification and quantification of five quinolones in urine samples directly injected into a micellar liquid chromatographic system without any pre-treatment step. Since the simultaneous elution of the five compounds was not resolved, two mobile phases have been proposed: (a) for ciprofloxacin and levofloxacin 0.15 M sodium dodecyl sulphate, 12.5% propanol and 0.5% triethylamine at pH 3.0 as the mobile phase and the detector at excitation wavelength 285 nm and emission wavelength 465 nm; and (b) for lomefloxacin, ofloxacin and moxifloxacin 0.05 M sodium dodecyl sulphate, 12.5% propanol and 0.5% triethylamine at pH 3.0 as the mobile phase and the detector at excitation wavelength 295 nm and emission wavelength 485 nm. Using these conditions, and in accordance with the food and drug analysis (FDA) guideline, the limit of quantification was 1 ng/mL, and the relative standard deviation and accuracy of the inter-day assay were 1.0–8.4% and 0.11–1.5%, respectively. Detection of the urinary excretion of four quinolones was followed up at 12 h after the healthy volunteers had taken the drug. No potential interference from metabolites was observed. This procedure permits the rapid and reproducible measurement of low levels of quinolones in a small amount of urine.

1. Introduction

Quinolones are among the most important antibacterial agents used in human medicine. They are active against both Gram-positive and Gram-negative bacteria through the inhibition of their DNA gyrase [1], are mainly used in the treatment of human and veterinary diseases, and are also very useful in preventing diseases in animals [2,3]. There is concern about the possibility of exposure to low levels of these compounds resulting in the development of resistance of human pathogens to antibiotics [4,5].

Several chromatographic methods have been reported for the determination of these compounds. High performance liquid chromatography (HPLC) has become an important tool for the routine determination of quinolones [6]. Several references about the determination of different quinolones by using HPLC with UV and/or fluorescence detection have been recently reported in biological fluids [7–11], food [12–14] and the environment [15,16]. Novel fluoroquinolones, such as moxifloxacin, have been also analysed [17]. Chromatography coupled with ionization mass spectrometry has been reported for the analysis of quinolones in urine [18] and food [19,20]. Usually, it is a fast technique, but it requires complicated and expensive equipment, and a labour-intensive sample preparation procedure. Recently, capillary electrophoresis (CZE) methods have been developed for quinolones in biological and environmental samples [21,22].

Micellar liquid chromatography (MLC) is an attractive alternative to conventional HPLC methods using aqueous-organic mobile phases for the determination of drugs in physiological fluids with direct injection. MLC allows the analysis of complex matrices, usually without the aid of extraction [23,24] because micelles tend to bind proteins competitively, thereby releasing protein-bound drugs and proteins, thus considerably reducing the cost and analysis time. Proteins are solubilised in micellar media and washed harmlessly away to elute with the solvent front rather than precipitating into the column In addition, MLC has proved to be a useful technique in urine analyses [25–28].

The aim of this work is the application of a rapid, sensitive and selective reversed-phase HPLC procedure with micellar mobile phases to determine ciprofloxacin, levofloxacin, ofloxacin, lomefloxacin and moxifloxacin (Fig. 1) in urine samples using direct injection into two different sets of analytical conditions. The method has been validated according to Food and Drug Analysis (FDA) guidelines [29]. The procedure developed herein could also

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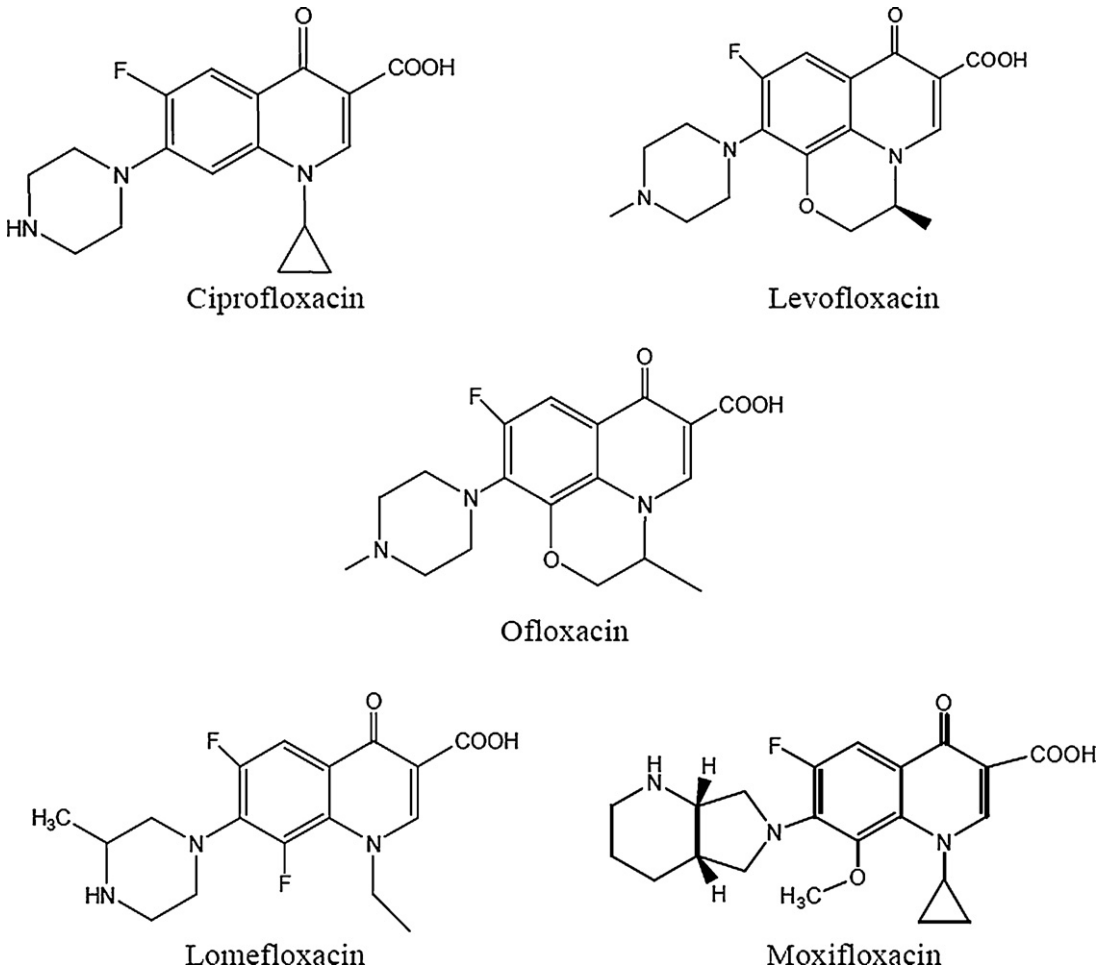

Fig. 1. Structures of the studied quinolones.

be useful in the area of quality control, routine analyses and pharmacokinetic studies.

2. Experimental

2.1. Reagents

Levofloxacin (LEV), Ciprofloxacin (CIP), Lomefloxacin (LOM), and Ofloxacin (OFL) were purchased from Sigma (St. Louis, MO, USA). Moxifloxacin (MOX) was purchased from Bayer (Leverkusen, Germany). Sodium dodecyl sulphate (SDS) and sodium hydroxide were acquired from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate and propanol were obtained from Scharlab (Barcelona, Spain). Hydrochloric acid, methanol, ethanol and triethylamine (TEA) were acquired from J.T. Baker (Deventer, the Netherlands). Ultrapure water was used throughout (Millipore S.A.S., Molsheim, France).

2.2. Apparatus and chromatographic conditions

The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, thermostatted autosampler tray and column compartments, and a fluorescence detector. The pH of the solutions was measured with a pH meter equipped with a combined Ag/AgCl/glass electrode (Crison GLP 22, Barcelona). The balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland). The vortex shaker and sonification unit were from Selecta (Barcelona).

The Agilent ChemStation (Rev. B.03.01) software was used for instrumental control and for chromatographic data collection. The Michrom software [30] was used for processing the chromatographic data and for optimisation studies.

The analytical separation was performed in a reversed-phase Kromasil C_{18} (Scharlab) (150 mm × 4.6 mm, 5 μm particle size). Two mobile phases were employed to analyse the quinolones (a) 0.15 M SDS, 12.5% propanol and 0.5% TEA at pH 3.0 for CIP and LEV; and (b) 0.05 M SDS, 12.5% propanol and 0.5% TEA at pH 3.0 for LOM, OFL and MOX. The retention times were lower than 10 min and 22 min, respectively. Detection was performed with a fluorescent detector set at the following excitations and emission wavelengths: 285/465 nm for the first group and 295/485 nm for the second group of quinolones, respectively. The flow rate and injection volume were 1 mL/min and 20 μL, respectively. Chromatographic experiments were carried out at laboratory temperature.

2.3. Standard, samples and mobile phase preparation

A stock solution of 10 μg/mL of the five quinolones was prepared by dissolving the compounds in a few millilitres of ethanol, with the aid of an ultrasonic bath, and was finally filled up with 0.05 M SDS at pH 3.0. Urine samples were collected in Urine Collection Cups (BD Vacutainer Systems, Plymouth, UK). Two spiked stock solutions, also containing 10 μg/mL of the both groups of compounds, were prepared by dilution of 1 mL urine in a factor (1:25) with 0.05 M SDS at pH 3.0. Solutions were

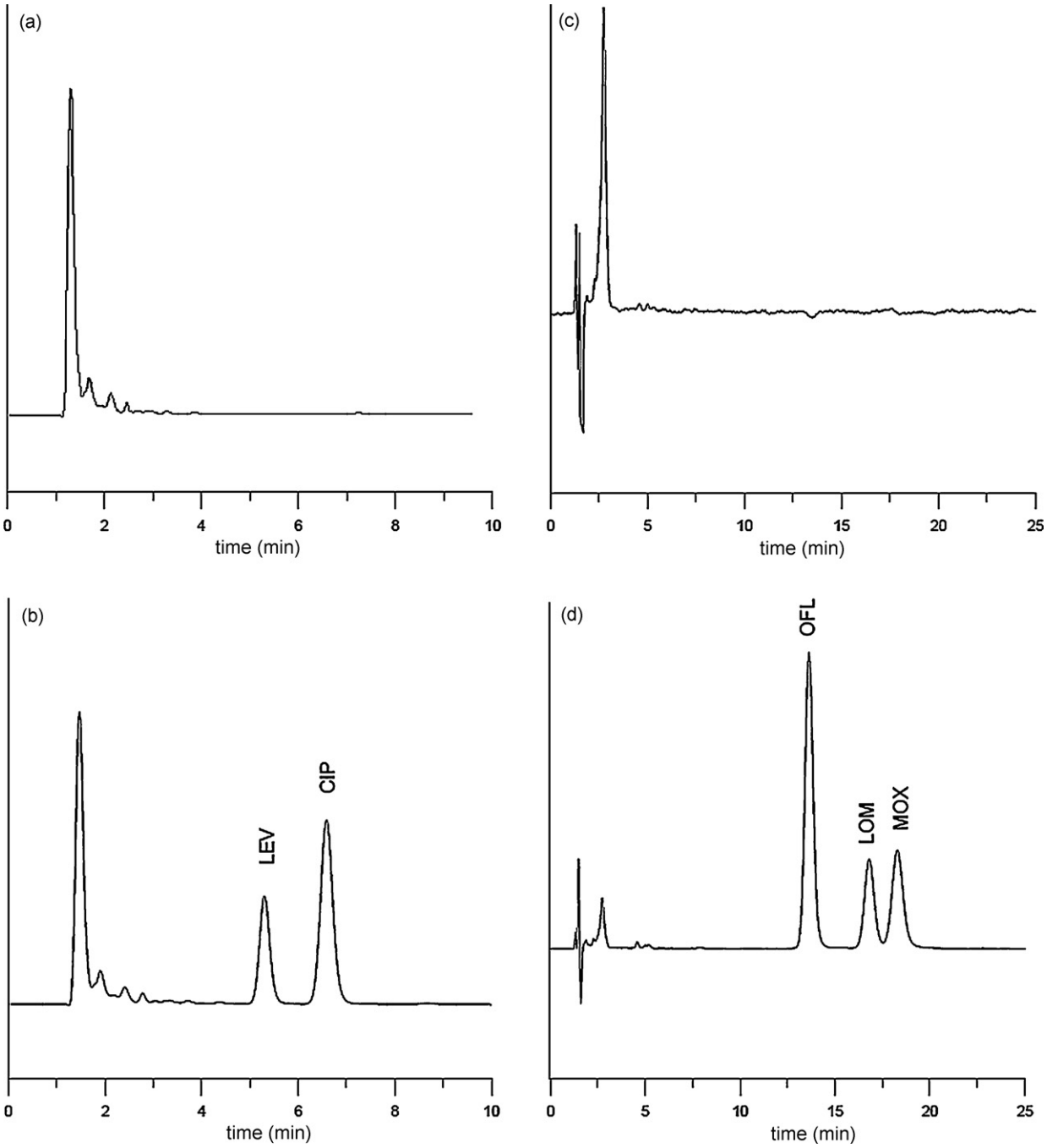

Fig. 2. Chromatograms of (a) urine blank, (b) urine spiked (100 ng/mL) with LEV and CIP using the mobile phase: 0.15 M SDS–12.5% propanol–0.5%TEA at pH 3.0 at FLD wavelength (285/465) nm; and (c) urine blank and (d) urine spiked (500 ng/mL) with OFL, LOM and MOX using the mobile phase: 0.05 M SDS–12.5% propanol–0.5%TEA at pH 3.0 at the FLD wavelength (295/485) nm. All the samples were diluted in a factor (1:25) with 0.05 M SDS.

prepared daily. The analyses of patients' urine were performed by diluting urine with 0.05 M SDS at pH 3.0 in different dilution factors, which was injected directly into the chromatographic system. All the solutions were filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA) before analysis.

The micellar mobile phases were prepared by dissolving SDS in water, which were buffered with sodium dihydrogen phosphate 0.01 M at pH 3.0 using 0.1 M of hydrochloric acid. A small amount (0.5%) of TEA was added to increase the peak efficiencies. Then, propanol was added to achieve the desired concentration. Finally water was added up to the mark-up of the volumetric flask.

3. Results and discussion

3.1. Optimisation strategy and mobile phase selection

Several studies were carried out to select efficient parameters for the analysis. Propanol yielded better efficiencies but larger retention times than butanol. However, the compounds could not be resolved from the protein band with butanol. Thus, propanol was preferred to optimise the separation of the compounds. Moreover, pH 3.0 was chosen because of the good retention times obtained with narrow and good efficiency. An amine (usually triethylamine, TEA) was used as a component of the mobile phase to protect silanol groups of the stationary

phase and to increase the peak efficiencies of the basic compounds.

The optimisation protocol began with the experimental design used for the drugs which consisted of five mobile phases: four located at the corners of a rectangular factor space and the fifth in its centre. The second step involved fitting the retention data to an adequate model (Eq. (1)) which, for the considered example, had four parameters. Errors below 3% were usually obtained for the prediction of the retention factors.

$$k = \frac{K_{AS}(1/1 + K_{AD}\varphi)}{1 + K_{AM}(1 + K_{MD}\varphi/1 + K_{AD}\varphi)[M]} \quad (1)$$

where [M] and φ are the concentrations of the surfactant and modifier; K_{AS} and K_{AM} correspond to the equilibria between the solute in bulk water and the stationary phase or micelle, respectively; K_{AD} and K_{MD} measure the relative variation in the concentration of the solute in bulk water and micelles due to the presence of a modifier, as compared to a pure micellar solution (without a modifier).

In order to find the best composition of the mobile phase, the five quinolones were injected into the mobile phases at pH 3.0, which contained SDS (M)/propanol (%, v/v): (0.05/2.5, 0.05/12.5, 0.1/7.5, 0.15/2.5, and 0.15/12.5), and the usual behaviour in MLC with SDS was observed. Thus, the retention factors decreased for SDS and propanol while increasing the concentration of both. On the other hand, efficiencies decreased when the surfactant concentration increased; conversely efficiencies increased with the higher concentrations of the modifier. Interpretative optimisation strategies can be assisted by computer simulation, which can mimic the methodology being followed by experienced chromatographers with less time and effort. We selected the most convenient mobile phase with the aid of the Michrom software by taking into account the factor of maximum resolution and the minimum analysis time [30]. This software allows for the graphic observation of the changes in the chromatograms when the user progressively varies the concentrations of the surfactant and modifier.

The simultaneous elution of the five drugs with the same mobile phase was firstly considered, but it was not possible due to an overlapping between CIP, LOM and MOX on the one side, and LEV and OFL on the other side. Then, the quinolones have been finally gathered in two different mobile phases. Moreover, quinolones do not present the same fluorescent maximum for excitation as they do for emission. Quinolones were divided into the two following groups: (a) CIP and LEV: mobile phase: 0.15 M SDS, 12.5% propanol and 0.5% TEA at pH 3.0. Fluorescence detector: excitation wavelength: 285 nm, emission wavelength 465 nm; and (b) OFL, MOX and LOM: mobile phase: 0.05 M SDS, 12.5% propanol and 0.5% TEA at pH 3.0. Fluorescence detection: excitation wavelength 295 nm, emission wavelength 485 nm. Fig. 2b and d shows the chromatograms for both groups.

Table 1
Linear regression data and the limits of detection.

Analyte	Slope ± SD	Intercept ± SD	r^2	LOD (ng/mL)
Ciprofloxacin	0.0101 ± 0.0004	−0.0226 ± 0.0021	0.99997	0.3
Levofloxacin	0.0073 ± 0.0005	−0.0055 ± 0.0021	0.99997	0.2
Lomefloxacin	0.0117 ± 0.0005	−0.0022 ± 0.0022	0.99998	0.4
Moxifloxacin	0.0060 ± 0.0004	−0.006 ± 0.004	0.99996	0.5
Ofloxacin	0.0158 ± 0.0003	−0.006 ± 0.008	0.99998	0.4

The chromatographic parameters (retention factor (k), efficiency (N) and asymmetry factor (B/A)) for the compounds of the first group (CIP and LEV) were: 4.6, 1950 and 1.1 and 3.5, 1836 and 1.1, respectively. And the chromatographic parameters for the second group (LOM, MOX and OFL) were: 13.8, 2447 and 1.2; 15.0, 1650 and 1.4; and 11.0, 2384 and 1.2, respectively.

3.2. Method validation

Method validation was done following the FDA validation guide [29]. The parameters evaluated were: linearity, detection and quantification limits, precision and accuracy, selectivity, recovery and robustness.

3.2.1. Selectivity

Six selected control drug-free human urine samples were processed directly into the chromatographic system and analysed to determine the extent to which endogenous components may contribute to interfere with the retention time of the drug. No interference for endogenous compounds was found in the physiological matrix studied, as Fig. 2a and c depicts.

3.2.2. Linearity

Calibration curves were constructed using the areas of the chromatographic peaks obtained at eight different concentrations (six replicates), in the range of 1–1000 ng/mL in the urine matrix solution (1:25 dilution factor). To study the variability of the calibration parameter, curves were obtained for 5 days over a 2-month period

Table 2
Inter-day and intra-day precision and accuracy of analytes.

Analyte	Concentration added (ng/mL)	Found[a] (mean ± SD) (μg/mL)	Accuracy (%)	Intra-day RSD (%)	Found[b] (mean ± SD) (ng/mL)	Accuracy (%)	Inter-day RSD (%)
Ciprofloxacin	5	5.02 ± 0.20	0.4	4.0	4.99 ± 0.06	0.2	5.6
	50	50.3 ± 1.6	0.5	3.15	50.1 ± 0.5	0.2	3.6
	500	498 ± 7	0.5	1.4	509.3 ± 2.1	0.4	3.3
Levofloxacin	5	5.01 ± 0.07	0.12	1.4	5.00 ± 0.05	0.2	8.2
	50	50.4 ± 1.1	0.7	2.1	49.8 ± 0.4	0.6	7.0
	500	499.8 ± 5.4	0.04	1.1	499 ± 3	0.7	6.7
Lomefloxacin	5	5.0 ± 0.3	0.4	6.8	5.01 ± 0.04	0.1	4.1
	50	49.3 ± 0.7	1.4	1.3	49.41 ± 0.24	1.2	4.6
	500	496.89 ± 0.13	0.03	1.1	499.5 ± 2.4	0.1	3.9
Moxifloxacin	5	4.91 ± 0.16	1.9	3.2	5.00 ± 0.12	0.04	8.4
	50	50.1 ± 1.7	0.3	3.4	50.0 ± 0.6	0.04	6.1
	500	497 ± 5	0.6	1.0	497 ± 4	0.6	6.7
Ofloxacin	5	5.1 ± 0.3	1.7	5.9	5.04 ± 0.03	0.8	7.8
	50	51.2 ± 0.3	2.5	0.5	50.8 ± 0.4	1.5	1.0
	500	496 ± 5	0.7	1.0	497.8 ± 2.1	0.4	1.7

[a] $n = 6$.
[b] $n = 5$.

Table 3
Robustness evaluation of the MLC method.

Chromatographic changes		Level	Ciprofloxacin[a]		Levofloxacin[a]		Ofloxacin[b]		Lomefloxacin[b]		Moxifloxacin[b]	
			t_R (min)	Area	t_R (min)	Area	t_R (min)	Area	t_R (min)	Area	t_R (min)	Area
(A) SDS concentration (M)												
(a) 0.145	(b) 0.045	−0.05	7.61	4.66	6.02	3.65	15.2	8.19	18.16	6.17	20.91	2.90
0.15	0.05	0	6.61	4.10	5.4	3.66	13.7	7.61	16.8	5.51	18.48	2.75
0.155	0.055	+0.05	6.28	4.66	5.23	3.12	13.4	7.57	16.7	5.84	17.94	2.64
Mean ± SD			6.8 ± 0.7	4.5 ± 0.3	5.5 ± 0.4	3.5 ± 0.3	14.1 ± 0.9	7.8 ± 0.3	17.2 ± 0.8	5.84 ± 0.3	19.1 ± 1.6	2.76 ± 0.13
RSD (%)			10.18	7.15	7.6	8.9	6.8	4.5	4.6	5,6	8.3	4.9
(B) Percentage of propanol (v/v)												
12.4		−0.1	6.74	4.06	5.04	3.32	16.18	7.34	17.9	5.44	18.72	2.71
12.5		0	6.61	4.10	5.4	3.66	13.7	7.61	16.8	5.51	18.48	2.75
12.6		+0.1	6.68	3.57	5.25	3.23	13.53	8.32	16.47	5.76	17.53	2.87
Mean ± SD			6.68 ± 0.07	3.9 ± 0.3	5.34 ± 0.08	3.40 ± 0.23	14.5 ± 1.5	7.8 ± 0.5	17.1 ± 0.7	5.57 ± 0.17	18.2 ± 0.6	2.78 ± 0.08
RSD (%)			1.03	7.5	1.5	6.6	10.3	6.5	4.4	3.0	3.4	2.9
(C) pH of mobile phase												
2.9		−0.1	6.72	3.58	5.41	3.98	14.18	7.48	17.93	5.44	19.18	2.76
3		0	6.61	4.10	5.4	3.66	13.66	7.61	16.8	5.51	18.48	2.75
3.1		+0.1	7.05	3.39	5.65	3.23	13.53	7.84	17.54	5.64	18.71	2.96
Mean ± SD			6.79 ± 0.23	3.7 ± 0.4	5.47 ± 0.15	3.85 ± 0.17	14.2 ± 0.5	7.64 ± 0.18	17.4 ± 0.5	5.53 ± 0.10	18.8 ± 0.4	2.82 ± 0.12
RSD (%)			3.4	5.5	2.8	4.5	3.7	2.3	3.7	1.8	3.7	4.14
(D) Flow rate (mL/min)												
0.9		−0.1	7.50	3.58	6.04	3.79	15.71	6.85	19.48	6.66	21.08	2.83
1		0	6.61	4.10	5.4	3.66	13.7	7.61	16.8	5.51	18.48	2.75
1.1		+0.1	5.99	3.39	4.83	3.10	12.67	6.36	15.7	5.71	17.03	2.51
Mean ± SD			6.7 ± 0.8	3.7 ± 0.4	5.4 ± 0.6	3.5 ± 0.4	14.0 ± 1.5	3.5 ± 0.4	17.3 ± 1.9	5.9 ± 0.6	18.9 ± 2.1	5.9 ± 0.6
RSD (%)			11.3	10.01	11.24	10.5	11.04	9.01	11.23	10.31	10.88	6.15
(E) Percentage of TEA (v/v)												
0.45		−0.05	6.66	4.54	5.35	3.45	14.6	7.75	18.6	5. 88	18.62	2.79
0.5		0	6.61	4.10	5.4	3.66	13.7	7.61	16.8	5.51	18.48	2.75
0.55		+0.05	6.73	4.20	5.38	3.38	13.6	7.73	16.1	5.79	18.20	2.71
Mean ± SD			6.66 ± 0.06	4.28 ± 0.23	5.36 ± 0.01	3.50 ± 0.14	14.0 ± 0.6	7.70 ± 0.08	16.8 ± 0.7	5,7 ± 0.19	18.44 ± 0.21	2.75 ± 0.03
RSD (%)			0.9	5.3	0.23	4.12	4.22	1.01	4.0	3.4	1.15	1.23

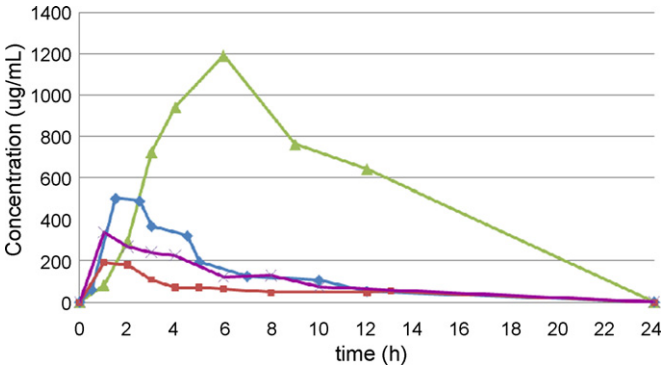

Fig. 3. Elimination curve of CIP (♦), LEV (▲), MOX (■) and OFL (×) in urine.

for a different set of standards. The slope and intercept were determined by the least squares linear regression analysis method. The results obtained are shown in Table 1. The linear correlation coefficients (r) were always higher than 0.99996.

3.2.3. Detection and quantification limits

The limit of detection (LOD) and the limit of quantification (LOQ) are obtained according to the FDA validation guide [29]. The LOD for the five quinolones in urine ($n = 10$) was determined with the *3s criterion* using a series of 10 solutions containing a low concentration of each compound (0.5 ng/mL). The LOQ in urine was selected as the lowest concentration used in the calibration curve. The LODs of CIP, LEV, MOX, OFL and LOM in urine-SDS solution were lower than 0.5 ng/mL (Table 1), while the LOQs were 1 ng/mL. It should be noted, both LODs and LOQs were in good agreement with the therapeutic levels in urine of these antibiotics.

3.2.4. Precision and accuracy

The intra- and inter-day precisions of the method were determined by analysing the five quinolones at three different concentrations (5, 50 and 500 ng/mL) in urine–SDS (1:25). The intra-day analyses were determined by injecting these three test solutions six times on the same day. The inter-day analysis was the average of six measurements of the intra-day values taken on 5 days over a 3-month period performed by different analysts and equipment at the same concentrations. The results, expressed as the percentage of the relative standard deviation and relative error (accuracy, %) for the intra- and inter-day values, are provided in Table 2. As seen, all the quinolones could be easily determined at the three concentration levels, and the obtained recoveries were quantitative in all cases with RSDs values lower than 8.4%. These results prove that the proposed method is suitable for the analysis of these antibiotics in urine samples. Thus, the procedure developed can be used in the quality control, routine analyses and pharmacokinetic studies.

3.2.5. Robustness

The robustness of the method was examined by replicate injections ($n = 6$) of a standard solution at 500 ng/mL with slight variations made to the chromatographic parameters (surfactant concentration, percentage of propanol, pH, percentage of triethylamine and flow rate). Negligible differences in the peak areas and less variability in the retention time were observed. The results, shown in Table 3, indicate that the selected factors remain unaffected by the slight variations made to these parameters. As expected, the variation of the flow rate shows the strongest influence on the retention of the studied compounds, unlike the other parameters.

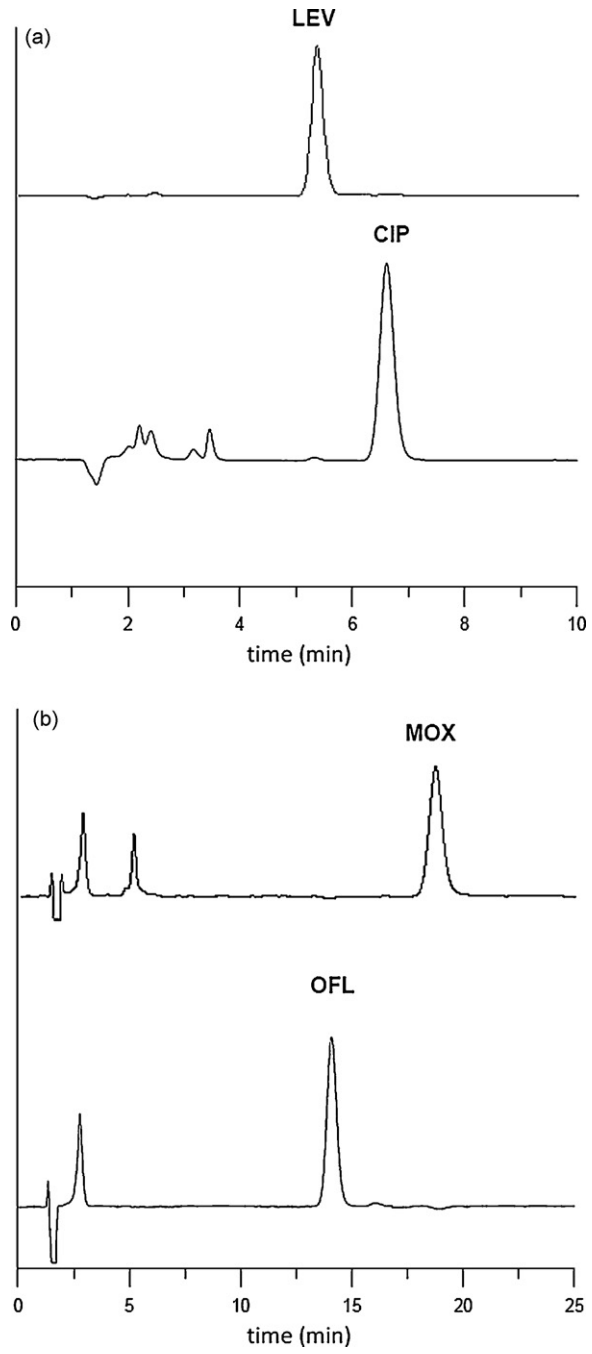

Fig. 4. Chromatograms of (a) CIP and LEV excreted in urine as unchanged drug 3 h after oral ingestion. Mobile phase: 0.15 M SDS–12.5% propanol–0.5%TEA at pH 3.0; and (b) OFL and MOX excreted in urine as unchanged drug 3 h after oral ingestion. Mobile phase: 0.05 M SDS–12.5% propanol–0.5%TEA at pH 3.0.

3.2.6. Recoveries

The quinolone recoveries from urine were determined by spiking drug-free urine diluted in a 1:25 factor with 0.05 M SDS at pH 3.0 with known amounts of the drug at five different concentrations (5–1000 ng/mL) within the calibration range (six replicates for each standard). The spiked samples were processed and analysed with the developed procedure. The relative (analytical) recovery was calculated by comparing the concentration obtained from the drug-supplemented urine with the actual added amounts. The results obtained show satisfactory recoveries for the five quinolones in the range of 96–103%. Fig. 2b and d depicts the chromatograms

obtained from the two groups of compounds in urine eluted in their optimum mobile phase.

3.2.7. Analysis of real urine samples

To determine the reliability of the assay, four urine samples from different volunteers were used. The matrix samples (in absence of quinolones) were chromatographed using the proposed procedure without any other treatment except filtration. Urinary studies were conducted following the oral administration of a single dose of a conventional tablet containing LEV (500 mg), CIP (500 mg), MOX (400 mg) and OFL (200 mg) to four different volunteers. A sample was collected just before administering the drug to be used as the blank. The other urine samples were collected at appropriate time intervals post-dose, approximately every 1 h, and were protected from light and refrigerated at 4 °C until analysed. Different dilutions of the urine samples were performed. The dilution factors for LEV, CIP, OFL and MOX were 0.025:25, 0.0125:25, 0.025:25 and 0.25:25, respectively, using 0.05 M SDS at pH 3.0. Fig. 3 shows the urine concentration profile of the four quinolones obtained.

The maximum concentration excreted in urine was found 6 h, 2 h, 1 h and 1 h after orally administering LEV, CIP, MOX and OFL, respectively. The final quantity of unchanged drug eliminated after 12 h was 448 mg (89%) for LEV, 290 mg (58%) for CIP, 92 mg (23%) for MOX, and 163 mg (82%) for OFL of the dose taken. These results are in accordance with the information found in the literature [31], which indicates that the quinolones excreted mainly as the unchanged drug in urine were 80–85% for LEV, 40–60% for CIP, 20% for MOX and 80% for OFL. Fig. 4 shows the chromatogram of (a) CIP and LEV, and (b) OFL and MOX excreted 3 h after oral administration. The peaks which could be assigned to the metabolites of the antibiotic or degradation products did not interfere in any analysis. All the compounds could be still detected up to 12 h after ingestion. These results show that pharmacokinetic studies can be performed under the proposed chromatographic conditions.

4. Conclusions

This assay, which has been designed to achieve high throughput samples in a short time for the preparation step, could be used to confirm and quantify urine samples originating from the screening process. The proposed chromatographic procedure provides good results for the determination of quinolones in urine in terms of linearity, accuracy, recoveries and robustness. Other commonly administered drugs do not interfere and the limit of detection is at the ng/mL level. This means that the proposed procedure is particularly useful for pharmacokinetic studies with healthy subjects and patients using small volumes of urine samples.

This method seems to be more sensitive than those reported previously [10,18,21]. Besides, according to the other methods, the retention time is quick enough for routine analysis [9,10,21,22]. Moreover, the proposed chromatographic procedure is also simpler than most methods reported where a previous extraction and/or an internal standard is required [9,18,21].

In conclusion, our results indicate that the MLC procedure can be used for the analysis of five quinolones, which are frequently prescribed drugs, in urine samples. Moreover, this method is sensitive enough to undertake the quality control routine analyses and pharmacokinetic studies of the drug, bearing in mind that the urine samples were injected without any previous treatment.

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