Determination of Ciprofloxacin and Levofloxacin in Human Sputum Collected from Cystic Fibrosis Patients using Microextraction by Packed Sorbent-High Performance Liquid Chromatography PhotoDiode Array Detector

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Abstract

This paper reports a new, easy, cheap, and fast MEPS-HPLC-PDA method for the simultaneous analysis of ciprofloxacin and levofloxacin, two fluoroquinolones (FLQs) commonly used for the treatment of pulmonary infections in cystic fibrosis (CF) patients. The FLQs were resolved on a Discovery C_8 column (250 mm × 4.6 mm; 5 μm particle size) using an isocratic elution with a run time of 15 minutes, without further purification. The method was validated over concentrations ranging from 0.05 to 2 μg/mL for both analytes in human sputum, and Enrofloxacin was used as internal standard.

This method was successfully tested to detect FLQs in sputum collected from CF patients. The MEPS-HPLC-PDA method was validated using biological samples collected from CF patients orally or intravenously injected with FLQs. The resultant data showed that the method is selective, sensitive and robust over range of concentrations for both FLQs. The limit of quantification of the method was 0.05 μg/mL for both analytes (comparable to more complex and expensive instrument configurations), weighted-matrix-matched standard curves showed a good linearity up to 2 μg/mL, and parallelism tests were also successfully assessed. The intra- and inter-day precision (RSD%) values were ≤10.4% and ≤11.1%, respectively, for all range of analysis. The intra- and inter-day trueness (Bias%) values are ranged from −11.8% to 7.25% for both antibiotic drugs.

At the best of our knowledge, this is the first MEPS-HPLC-PDA based method that uses MEPS procedure for simultaneous determination of ciprofloxacin and levofloxacin in human sputum. The method was tested successfully on real sputum samples by following a conventional drug administration. Furthermore, the MEPS-HPLC-PDA based method provides more advantages to detect and analyze quickly the antibiotic drugs in biological matrices than other analytical procedures reported in literature.

Keywords: MEPS-HPLC-PDA; Method development; Levofloxacin and Ciprofloxacin; Sputum; Sample preparation; Cystic fibrosis.

Introduction

The biological samples cannot easily analyzed due to multiple components forming their constituents. Although, several analytical methods can be used to resolve drugs and metabolites from serum, plasma and urine, poor informations are actually available to detect drugs and metabolites in sputum. In this attempt, the high performance extraction procedures and separation techniques provide an accurate quantification of drugs and metabolites at low concentrations and allow to detect selectively these compounds from biological samples. In particular, we previous demonstrated that the high performance liquid chromatography (HPLC) [1-3] and chemometric analysis [4], combined with specific instrument set-up [5], extraction procedures [6] and/or multi-factorial analyses [7-9], can increase the detection of drugs and metabolites in biological samples, thus improving the accuracy and specificity of analysis. This is an important requirement particularly for antibiotic drugs, which are metabolized rapidly after oral and/or systemic injections and need high therapeutic dosage at targeted tissues. These requirements play a pivot role for the industrial pharmacy and clinical laboratory to easy and cheap develop analytical methods to detect simoultaneously antibiotic drugs without employing techniscians with great expertise in analytical chemistry.

The ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) (**Figure 1**) is the most potent second-generation fluoroquinolones (FLQs) *versus* gram-negative bacteria, exhibiting a rapid onset of action without cross-reaction with penicillin, cephalosporins, and aminoglycosides [10]. By comparing to different FLQs, the ciprofloxacin inhibits the DNA gyrase (topoisomerase II) and lacks the DNA replication.

The levofloxacin ((S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid) (**Figure 1**), is the L-isomer of the racemate Ofloxacin [11], and a third-generation FLQs, which shows a broad-spectrum activity versus both Gram-positive and Gram-negative bacteria. Furthermore, the levofloxacin also demonstrates higher activity versus Gram-positive bacteria than the ciprofloxacin [12].

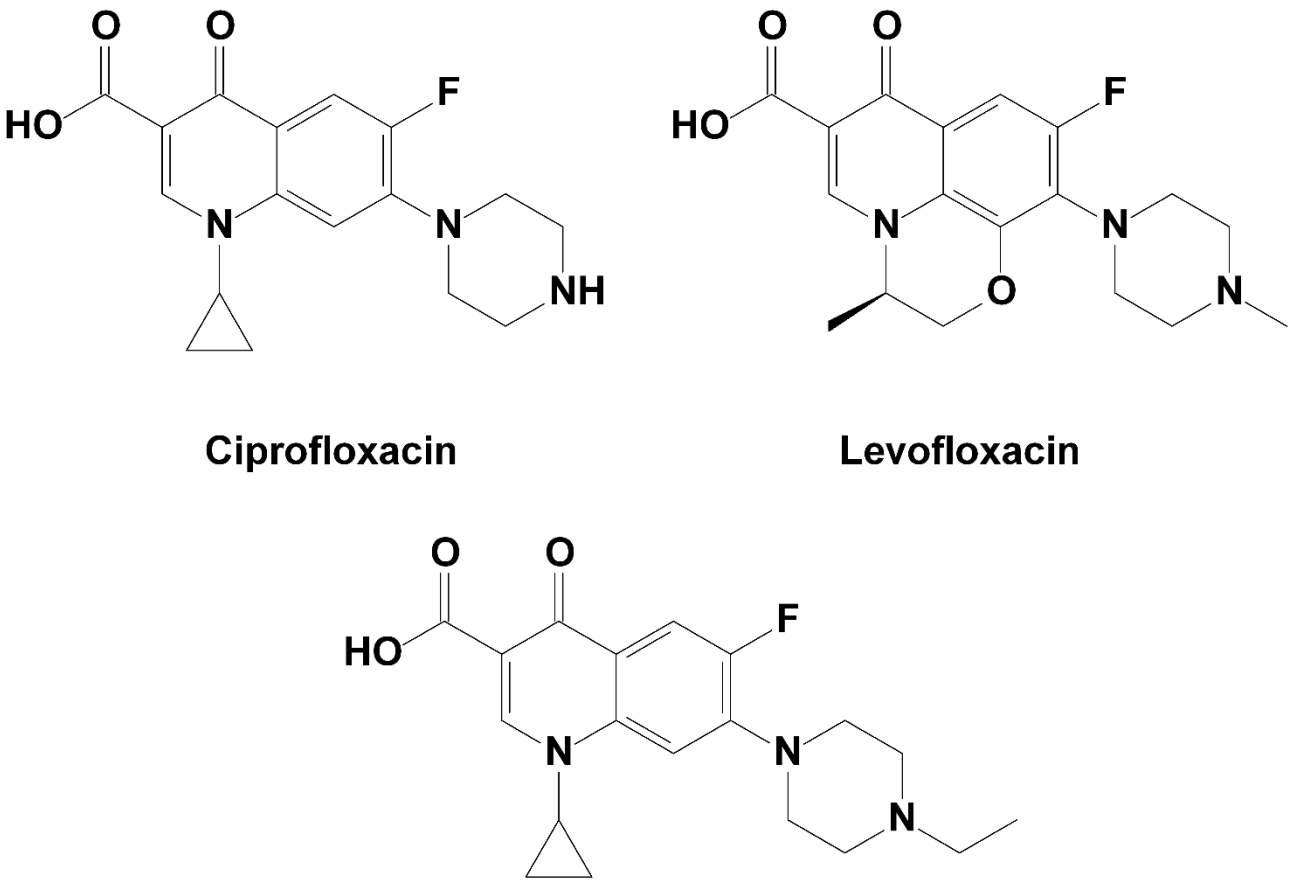


Figure 1. Chemical structures of Ciprofloxacin, Levofloxacin, and Enrofloxacin (IS).

The FLQs are often used to treat lung infections in cystic fibrosis (CF) patients. Particularly, Ciprofloxacin and Levofloxacin show a significant bactericidal activity versus *Pseudomonas aeruginosa*, which causes chronic lung infections in patients affected from CF and often produces a recurrent pulmonary exacerbation, which requires several antibiotic administrations [13]. Furthermore, the FLQs prevent of the pulmonary antibiotic-resistance occurred by biofilms both in *P. aeruginosa* [14] and other CF pathogens [15, 16].

The intravenous administration (IV) of therapeutic compounds can activate several metabolic pathways, which can metabolize antibiotic drugs and decrease their bioavailability and therapeutic dosage on the targeting tissues. Conversely, the aerosol injection can deliver high dosage of therapeutics at the pulmonary tissue and decrease their potential side effects due to the excess of

drugs, which are not adsorbed on lungs using the IV or oral (OS) administrations [17]. Although, the efficacy of therapeutic treatment of CF patients depends on the antibiotic drug concentrations into the pulmonary tissue [18], the detection of FLQ drug dosage plays a main role for the successful of therapy in CF patients administered IV or per OS. Particularly, the dosage of FLQs needs to be detected into sputum and other biological fluids in order to customize therapy using ciprofloxacin and levofloxacin in patient affected from CF disease [19].

Basically, drugs and metabolites can be separated and easily detected from biological samples using chromatography. In particular, the High Performance Liquid Chromatography (HPLC) [20-23], the High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) [24], Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) [25] and other procedures [26, 27] can be used to quantify drugs and metabolites in plasma, serum, urine and pharmaceutical formulations as previously reported [28]. Conversely, no studies discuss actually the simultaneous detection and quantification of ciprofloxacin and levofloxacin in human sputum combining microextraction by packed sorbent (MEPS) procedure, which is a cheap, easy and recent technique to separate analytes and get higher extraction from biological samples and HPLC apparatus. In fact, Huang and colleagues report the determination of the Moxifloxacin in sputum collected from patients affected by non-CF pulmonary diseases [29]; while Myers and colleagues firstly quantify the ciprofloxacin in serum, urine and sputum by using high-performance liquid chromatography with fluorescence detection [30].

We previous demonstrated that analytical procedures allow to detect, separate and quantify drugs, metabolites and impurities from biological samples [31-36]. Furthermore, we already used microextraction by packed sorbent-high performance liquid chromatography-photodiode array (MEPS-HPLC-PDA) method to analyze simultaneously seven non-steroidal anti-inflammatory drugs, i.e. furprofen, indoprofen, ketoprofen, fenbufen, flurbiprofen, indomethacin, and ibuprofen, in human plasma and urine [6]. We report herein a MEPS procedure coupled to easy, cheap, rugged, well-known and routine instrument configuration (high performance liquid chromatography-

129 photodiode array, HPLC-PDA) to quantify simultaneously the commercially available ciprofloxacin
130 and levofloxacin drugs in sputum samples collected from CF patients administered IV or per OS
131 therapy. This procedure can provide several advantages to extract and detect analytes in biological
132 samples compared to the other expensive and complex instrument configurations.
133
134 **2. Experimental**
135 *2.1 Chemicals, solvents, and devices*
136 Levofloxacin, ciprofloxacin, enrofloxacin (used as Internal Standard, IS) (all >98% purity
137 index), sodium phosphate monobasic and sodium phosphate dibasic (>99% purity index), phosphoric
138 acid (to obtain phosphate buffer at pH = 2.5), and triethylamine (>99.5% purity index, TEA) were
139 purchased from Sigma-Aldrich (Milan, Italy). The commercially available levofloxacin (Tavanic®)
140 and ciprofloxacin (Ciproxin®) drugs were obtained from Sanofi-Aventis S.p.A. (Milan, Italy) and
141 Bayer S.p.A. (Milan, Italy), respectively.
142 Methanol and acetonitrile (AcN) (HPLC-grade) were purchased from Carlo Erba (Milan,
143 Italy) and were used without further purification. The water for HPLC analysis was generated by
144 Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).
145 Oasis HLB (1 cc, 30 mg) and Sep-Pak (1 cc, 50 mg) were purchased from Waters (Milford,
146 MA, USA); Evolute (1 cc, 25 mg) from Stepbio (Bologna, Italy); Strata-X (1 cc, 30 mg) from
147 Phenomenex (Torrance, CA, USA); and Bond Elut (1 cc, 50 mg) from Agilent (Santa Clara, CA,
148 USA).
149 MEPS device (syringe) and replacement needle with C_{18} stationary phase were purchased
150 from SGE Analytical Science (Australia).
151
152 *2.2 Sputum collection and storage*
153 Sputum samples were collected from CF patients hospitalized in Cystic Fibrosis Unit at the
154 "Bambino Gesù" Children Hospital of Rome. The patient was informed about all procedures before

a written informed consent was carried out. The samples were collected, by coughing into a sterile container, both from patients not undergoing FLQs therapy (controls) and those under treatment with ciprofloxacin or levofloxacin (each at 500 mg twice daily). All samples were stored at -80°C until further analysis.

2.3 Sputum sample preparation

180 μL of human blank sputum was mixed with 10 μL of analyte working solutions and 10 μL of IS (1 μg/mL), vortex-mixed for 1 minute (10% *v:v* of matrix modification for calibration curve and QC samples, 5% *v:v* of matrix modification for real samples).

A preliminary cleaning and dilution step for sputum samples was achieved using trichloroacetic acid (TCA) (20 mg/mL) in 1: 0.5 ratio (*v:v*) followed by centrifugation at 12.000 × *g* for 5 minutes, and the resultant samples were exctracted using MEPS device. This step denatures the proteins, hydrolyses the bound drug residues, and reduces the sample density to facilitate the sample flow through the C_{18} stationary phase, as previously described [37].

The MEPS device, consisting of a 250 μL syringe with a replacement needle, was used in *off-line* instrument configuration to separate and purify samples. The MEPS removable needle, containing a C_{18} stationary phase, was able to analyse at least 90-120 samples before it was changed. To enhance the overall process efficiency, the total volume of each step was achieved performing multiple "*suctions*" (in and out).

The extraction procedure was performed as herein reported: the sorbent was conditioned with 3 × 150 μL of methanol and 3 × 150 μL of phosphate buffer (30 mM, pH 2.5); sample application by passing the biological fluid (sputum) diluted 1:0.5 (*v:v*) with TCA (20 mg/mL) (8 × 100 μL) through the sorbent; wash step with 1 × 150 μL of phosphate buffer (30 mM, pH 2.5) and methanol (95:5, *v:v*); the analytes was eluted with 8 × 25 μL of methanol in a vial and then directly injected into the HPLC system. The average flow rate at every single step was 10 μL/s.

2.4 Apparatus and chromatographic condition

HPLC analyses were performed on a Waters liquid chromatography equipped with a model 600 solvent pump, and a 2996 photodiode array detector. Mobile phase was directly *on-line* degassed by using Degassex, mod. DG-4400 (Phenomenex, Torrance, CA, USA). Empower v.2 Software (Waters Spa, Milford, MA, USA) was used for data acquisition and analysis.

In order to optimize the chromatographic conditions, different columns were tested, such as Gemini C_{18} (250 × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, CA, USA), GraceSmart RP_{18} (250 × 4.6 mm, 5 μm particle size, Grace, Deerfield, IL, USA), and Discovery C_8 column (250 × 4.6 mm, 5 μm particle size, Supelco, Milan, Italy).

Discovery C_8 packing column (250 × 4.6 mm, 5 μm particle size; Supelco, Milan, Italy) connected to a Security Guard column (4.0 × 3.0 mm, 5 μm particle size; Supelco, Milan, Italy) was finally used to separate antibiotic drugs and IS. The column was thermostated at 25°C (± 1°C) using a Jetstream2 Plus column oven during the analysis.

For quantitative analyses, the selective detection was performed at 295, 279, and 278 nm for levofloxacin, ciprofloxacin, and enrofloxacin (IS), respectively (see Supplementary Material, section S.1 for analytes and IS UV/Vis spectra).

An isocratic elution mode was performed using a binary solvent system composed by phosphate buffer (30 mM, pH 2.5, 1% TEA), and AcN (1% TEA) (86:14, *v:v*) at 1.0 mL/min flow rate.

2.5 Stock solution, calibration curve and QC analysis

The three chemical standards stock solutions were made at the concentration of 1 mg/mL in a final volume of 10 mL of mobile phase. The combined working solutions of mixed standards at the concentrations ranging from 1 to 40 μg/mL were obtained by the dilution of a mixed solution at 500 μg/mL in volumetric flasks containing the mobile phase.

206 Finally, the eight calibration standards were carried out as previously reported (see Section
207 2.3 Sputum sample preparation) and injected into the HPLC-PDA system.
208
209 *2.6 Method validation*
210 In order to demonstrate the suitability of the developed analytical method, validation was
211 carried out according to International Guidelines [38-40]. In this way, Limit of Detection (LOD),
212 Limit of Quantification (LOQ), linearity, intra- and inter-day trueness and precision, selectivity,
213 recovery, stability, parallelism test, and ruggedness were tested for each analyte in sputum samples.
214 The method efficiency (recovery) was optimised to obtain the better results in terms of sample
215 clean up and maximum peaks area responses (as signal-to-noise ratio).
216
217 **3. Results and discussion**
218 *3.1 Optimization of MEPS extraction procedure*
219 A main step in the multi-drug determination procedure is represented by the extraction and
220 clean-up assays. Both allow to obtain the maximum recovery of analytes, without significant
221 interference peaks. Several assays were tested, starting from a simple Liquid-Liquid extraction (LLE)
222 with AcN [40], also acidified with phosphoric acid or TCA. These procedures allowed low recovery
223 for ciprofloxacin (approx. 60%), but gave higher values (approx. 80%) for levofloxacin, comparable
224 to those reported by Schulte and coworkers [41] for plasma samples.
225 The Solid Phase Extraction (SPE) procedure was also tested using different stationary phases
226 from different manufacturers, such as Oasis HLB, Evolute, Strata-X, Bond Elut, and Sep-Pak. All the
227 SPE sorbents were conditioned according to general procedures suggested by the manufacturer. These
228 procedures allowed to achieve high recovery values (approx. >80%), although high volumes of
229 samples were required.
230 Based on these results, a MEPS microextraction procedure was tested according to general
231 conditions for cartridge set up, sample extraction, and elution reported in literature [37].

For the MEPS optimisation, sputum QC samples supplemented with 0.15 μg/mL of analytes were used. Before the analysis, the biological samples are diluted in water at the ratio from 1: 4 to 1: 20 (*v/v*) and then centrifuged as previously reported [37]. The resultant sample was withdrawn and concentrated; while the cleaning step was previously achieved by using water (200 μL). The concentrated analytes on the stationary phase of MEPS were eluted using methanol (20 μL) and then directly injected into the HPLC-PDA apparatus. This procedure allowed for lower analyte signals and for the presence of interference peaks, but an overall improvement respect to initially tested procedure was observed in terms of signal-to-noise ratio. Additionally, the overall processing time is decreased from 30-40 minutes (LLE and SPE extractions) to approximatly 10 minutes.

The MEPS procedure was then optimised in order to better clean the sample from biological matrix interferences and to optimise the response in terms of peak area. In fact, the recovery procedure, which is expressed as a function of peak area, can increase linearly from single extraction cycle (1 × 100 μL) to eight cycles (8 × 100 μL) using "*draw–eject*" multiple extraction cycles from the same sample.

MEPS procedure was also optimized in order to improve the devices' lifetime to carry out multiple analysis; to decrease its cost and time consuming; and to prevent syringe-to-syringe variations. This "*target*" can lead to a not obvious analytical drawbacks, i.e. the carry-over. In fact, the carry-over represents a limiting step, compound-dependent phenomenon, and could be caused from the MEPS device, from the adsorption of analytes in the instrument under isocratic condition of the analysis. Furthermore, the carry over should be evaluated to increase precision and trueness of analytical method during the validation process.

The analyses were carried out using different tests. In particular, we firstly used ultra pure water (3 times) and elution solvent (4 times), during the washing step and sorbent cleaning, to decrease the carry-over below 0.02% [37]. Unfortunatly, the carry over was decreased but not suppressed during the analyses. Secondly, we changed the washing solvent and the ultrapure water was replaced with phosphate buffer (30 mM, pH 2.5) and methanol (95:5, *v:v*). This mixture improved

the signal-to-noise ratio, analytes recovery , and no carry-over. By changing the pH (2.5 – 7.4) and the ionic strength (5 – 50 mM) of phosphate buffer herein reported, we showed that a pH value of 2.5 prevents the arrangment of enrofloxacin in its zwitterionic form (pH 7.5); while a ionic strength of 30 mM increased the signal-to-noise ratio up to a plateau. The elution solvent was also optimized to increase the overall signal (in terms of analyte peak area) and improve the clean up of MEPS apparatus.

Pure methanol was used as elution solvent to perform the maximum overall recovery of analytes. Pure methanol as elution solvent was used in order to maximise the overall recovery. In this way the interactions between the analytes and the stationary phase were less strong leading to an increased response (in terms of analytes and IS peak area). In this attempt, we optimized the extraction procedure according to the following steps:

1. MEPS Conditioning: methanol (3 × 150 μL) and phosphate buffer (30 mM, pH 2.5) (3 × 150 μL);
2. MEPS Sample load: diluted sputum 1:0.5 (*v:v*) with TCA (20 mg/mL) (8 x 100 μL)
3. MEPS Wash: phosphate buffer (pH 2.5, 30 mM) and methanol (95:5, *v:v*) (1 × 150 μL)
4. MEPS Elution: methanol (8 × 25 μL)

This optimised procedure allowed to increase the sample cleaning up and maximum peaks area responses, to avoid the carry-over phenomena, and to reuse the device up to 90-120 folds without any loss of performance. The syringe-to-syringe variations were also tested. The accuracy of QC samples at three levels of concentrations (low, medium, high) was similar in terms of inter-day precision and trueness for all needles used during the analysis.

3.2 HPLC separation and method development

The chromatographic conditions were optimized to increase the drug detection and peak signals, to decrease the run time, and to avoid the presence of interferences during the analysis.

Different gradient and isocratic mobile phases were performed to separate accurately ciprofloxacin, levofloxacin, and IS.

The isocratic condition, made from different percentage of organic solvents and buffers at different pHs, were firstly carried out to develop a reproducible analytical method for HPLC analysis of ciprofloxacin and levofloxacin. The chromatographic separation of antibiotic drugs was performed using different stationary columns, i.e. two Octadecylsylane columns [41, 42], and one Octylsylane column: Gemini C_{18} (250 × 4.6 mm, 5 μm particle size), GraceSmart RP_{18} (250 × 4.6 mm, 5 μm particle size), and Discovery C_8 column (250 × 4.6 mm, 5 μm particle size). The mobile phase, made from organic solvent and buffer at different pHs, was following reported: (a) AcN and 2% *v:v* acetic acid [23], (b) 30 mM ammonium acetate buffer (pH 2.5) and AcN (80:20, *v:v*) to transfer the method from PDA to mass spectrometric detector, and finally (c) a binary solvent system, made from the sodium dehydrogenate phosphate buffer (30 mM, pH 2.5) with triethylamine (TEA) (1%, *v:v*), and AcN (84:16, *v:v*) with TEA (1%, *v:v*), using a flow rate of 1.0 mL/min.

The method (c) was used to separate ciprofloxacin, levofloxacin and IS during the analysis. In fact, it allows to separate drugs and IS accurately without any overlapping of drug retention times and interferences of sputum components during the MEPS extraction. The procedure reported in point (c) allows to detect ciprofloxacin, levofloxacin and IS at different retention times; to separate drugs and potential interference peaks derived from biological samples; and to maintain a suitable overall run time. Furthermore, to increase the peak shape and the retention times different TEA percentages, i.e. 0.5%, 0.8%, and 1% (*v:v*), was added to AcN and buffer phosphate during the analysis. The resultant data demonstrated that the peak shape of varios compounds is symmetric and the retention times of ciprofloxacin and levofloxacin, respectively, are well separated using 1% (*v:v)* of TEA. A slight asymmetric shape for the retention time occurred for IS using 1% (*v:v)* of TEA; however, this value does not affect significantly the peak integration during the analysis of data.

By applying the chromatographic condition herein reported, a robust baseline was carried out in 15 minutes to separate drugs, and the retention times of compounds were 8.38 (± 0.45), 9.19 (±

310 0.46), and 12.2 (± 0.53) for levofloxacin, ciprofloxacin, and enrofloxacin (IS), respectively [see
311 Supplementary Material, section S.2 for System Suitability Test (SST) separation].
312 The LOQ values were 0.05 μg/mL for levofloxacin and ciprofloxacin (based on signal-to-
313 noise ratio of 10:1, the analytes show a precision below 20% and a trueness over 80–120%),
314 respectively; while the LODs of the method was further set based on the signal-to-noise ratio (3:1) of
315 the chromatograms at 0.017 μg/mL for ciprofloxacin and levofloxacin, respectively (Table 1).
316

317 **Table 1:** Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent eight non-zero

318 concentration points.

319

Analyte	Linearity range (μg/mL)	Slope[a]	Intercept[a]	Detemination Coefficient (R^2)
Ciprofloxacin	0.05-2 (0.017 μg/mL[b])	0.157 (± 0.004)	-0.0002 (± 0.0005)	0.9933
Levofloxacin	0.05-2 (0.017 μg/mL[b])	0.192 (± 0.004)	0.0084 (± 0.0005)	0.9966

320 [a]Values at 95% confidence intervals on the mean of six independent calibration curves. [b]The round bracket shows the
321 LOD values obtained from signal-to-noise ratio (3); the slope and intercept of calibration curve are expressed in μg/mL.
322

The within-assay precision (repeatability) was carried out by performing six consecutive assays, on the same day, on QC samples spiked at three different drug concentrations, i.e. 0.15 (low level), 0.30 (medium level) and 0.75 (high level) μg/mL, which are within the range of the calibration curve. The QC samples were also analyzed in different days to evaluate the between-assay precision (intermediate precision). The trueness of the method was further tested using the same concentrations for ciprofloxacin and levofloxacin, respectively, and comparing the QC concentrations of both drugs with their nominal values (Table 2). The QCs over range at 40 μg/mL were also quantified after a dilution step (40 folds, *v:v*) with the pooled corresponding blank sputum matrix followed by MEPS-HPLC-PDA analyses, and the precision and trueness values were comparable to those obtained for low, medium, and high levels.

Table 2: Intra-day and Inter-day precision (RSD%), trueness (Bias%) of the analytical method obtained from the analysis of QC samples.

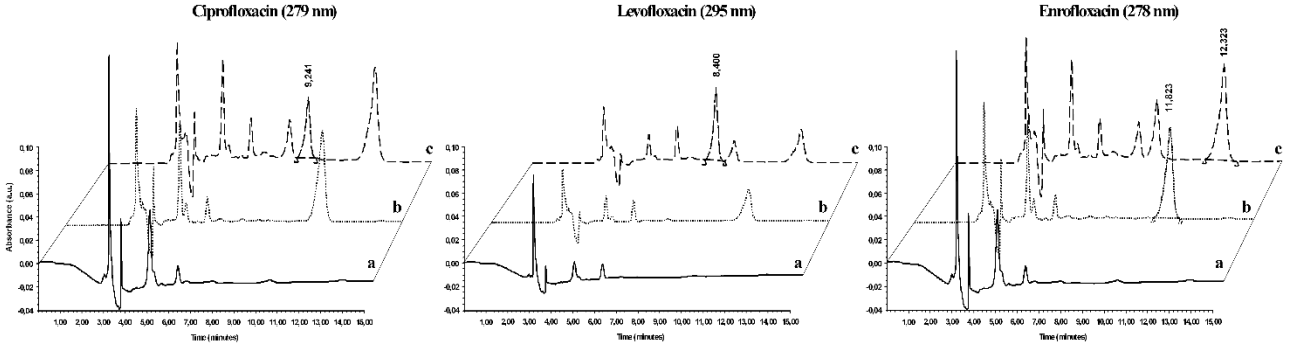
	INTRADAY		**INTERDAY**	
	Ciprofloxacin	**Levofloxacin**	**Ciprofloxacin**	**Levofloxacin**
Theoretical[a]		**0.15**		
Mean Back-Calculated[a]	0.16	0.15	0.15	0.16
BIAS%	7.25	0.12	-0.35	4.77
RSD%	6.32	9.18	6.27	5.70
Theoretical[a]		**0.30**		
Mean Back-Calculated[a]	0.27	0.29	0.27	0.29
BIAS%	-9.74	-3.81	-9.49	-3.76
RSD%	1.75	5.77	2.60	7.88
Theoretical[a]		**0.75**		
Mean Back-Calculated[a]	0.75	0.68	0.80	0.66
BIAS%	3.20	-4.35	6.82	-11.8
RSD%	6.09	11.1	10.4	3.19

The data are the mean values of six experiments (n = 6). [a]Concentration is expressed in μg/mL;

As reported in Figure 2, the selectivity and specificity of the method were tested on blank sputum samples extracted using MEPS procedure and analysed by HPLC-PDA apparatus without any fortification (a), and after the supplement of IS (b) or analytes plus IS (c). These expeimental

conditions demonstrated that the analyte retention times are similar to those of real samples and no interfering peaks were observed.

Figure 2. Chromatograms obtained after the extraction and analysis of ciprofloxacin, levofloxacin, and enrofloxacin at 279, 295, and 278 nm, respectively (trace a: blank human sputum, trace b: blank human sputum spiked with 1 μg/mL of Internal Standard, and trace c: blank human sputum spiked with 1 μg/mL of Internal Standard and 0.75 μg/mL of analytes). 20 μL of samples was injected during the analysis.



The carry-over was not obvious in the biological matrices, especially when isocratic elution mode was used. For this reason, a blank sputum sample was injected after the analysis of sputum fortified at the upper limit of quantification (ULOQ, 2 μg/mL) and no "*memory effects*" were observed. In addition, the intra-matrix variability was tested using six different batches of sputum samples, and no interferences for ciprofloxacin, levofloxacin and IS were carried out during the analysis.

3.3 Method validation

The calibration curves in blank sputum were calculated by analysing for six-times the eight non-zero concentration standards made in freshly spiked blank sputum, and the results were performed by plotting the corrected area (analyte area/IS area ratio) for each level versus the nominal concentration level corresponding to each standard solution. The linearity of the standard curves was assessed by calculating the intercept, slope, determination coefficient and variation in the range of

0.05-2 μg/mL for levofloxacin and ciprofloxacin, respectively. Levofloxacin and ciprofloxacin calibration samples were prepared by diluting their working standard solutions in sputum, and at least eight concentration levels were used. The calibration curves were linear over the range reported for a least-squares linear-regression determination coefficient (r^2) ≥ 0.9933, using a weighting factor of ($1/x^2$). The resultant calibration curves, for ciprofloxacin, levofloxacin and IS at their maximum wavelengths (295, 279, and 278 nm, respectively), were plotted using weighted linear least-squares regression analysis, as permitted by the method validation guidelines, stating that "*standard curve fitting is determined by applying the simplest model that adequately describes the concentration–response relationship using appropriate weighting...*" [38]. All calibration curve parameters are reported in Table 1.

The precision and trueness (also for QCs over range) were acceptable for RSD% and Bias% values below 15% (Table 2). The limit of quantitation was 0.05 μg/mL for both levofloxacin and ciprofloxacin (Table 2).

The selectivity of method was also studied by analysing six sputum samples from different patients. According to ICH guideline requires [39], the blank samples showed neither area values higher than 20% of LOQ areas at the analyte retention times, nor higher than 5% of IS area at the drug retention time. The representative chromatograms of antibiotic drugs obtained from simple human sputum (control) after extractions were shown in Figure 2 (trace a). No interfering peaks were detected at the levofloxacin or ciprofloxacin retention time (see Supplementary Material, section S.3).

No significant decrease of drug concentrations or changes of chromatograms, due to the potential degradation of antibiotic drugs, were carried out for the stock solutions, the spiked sputum samples and the extracts stored at room temperature. The spiked sputum samples stored at −20°C, at +4°C, and freezing-thawed samples (n = 3 cycles) were also stable for at least 1 month (see Supplementary Material, section S.4 for long-term stability, and Table S.4.1).

The HPLC-PDA method ruggedness was also carried out over chromatographic conditions that were designedly modified. Results supported the ruggedness of the developed method (Supplementary Material, section S.5, and Table S.5.1 for data on retention and selectivity factor).

A parallelism check was performed by analysing a high drug sputum sample concentration diluted 40-folds (*v:v*) with the pooled corresponding blank matrix used to make standards and QC samples. The resultant data demonstrated that a limit of quantification for sputum could be carried out by diluting samples up to 40 μg/mL above the maximum value of the standard calibration curve. The resultant values for the accuracy parameters, i.e. precision and trueness, can be compared to those obtained for the concentrations of antibiotic drugs within the calibration range.

3.4 Comparisons with existing methods

The resultant method showed several advantages to analyze simultaneously the antibiotic drugs. In fact, this procedure increases the detection of ciprofloxacin and levofloxacin in biological samples, particularly sputum collected from CF patients. The previous data showed that only single levofloxacin [17], ciprofloxacin [30], or other FLQs [29] was extracted from sputum collected from patients. Additionally, the methods previously reported require a complex instrument configuration of the HPLC and often a complex extraction procedure instead of MEPS-HPLC-PDA apparatus [23]. Conversely, the MEPS-HPLC-PDA method allows to detect ciprofloxacin and levofloxacin simultaneously in sputum samples of CF patients after a simple (similar to well-know SPE procedure but based on a syringe device), rapid, and selective MEPS extraction in approximatly 10 minutes without the presence of interfering peaks and/or the carry-over phenomena as reported in Materials and Methods section (3.3.2) and in Supplementary Materials section S.3. The resultant procedure allows the continuous analysis of samples under isocratic eluation at short running time (15 minutes, without re-conditioning HPLC apparatus); is not time consuming for the extraction of samples (10 minutes for MEPS apparatus) compared to other extraction procedure (20 minutes reported for the same analytes in other matrix [41]), does not require any further purification and/or extraction

process; and particularly, is a cheap (a single MEPS removable needle can be used to analyze almost 90-120 samples before its replacement) and green chemistry method.

The LOQs were carried out easely using MEPS-HPLC and PDA detector and were similar to those obtained using more sensitive detectors, i.e. FLD, which was commonly used to quantify drugs in plasma samples [42], more complex and/or expensive apparatus and equipment configurations, i.e. MIPs [43]. In particular, the LOQ values were included in the range from 2.5-3 [44, 45] to 100-folds lower than data previously reported [46], and were obtained using different biological samples and a more complex apparatus set up. The MEPS-HPLC-PDA also decreased of 5×10^4-folds the limit of detection for antibiotic drugs with respect to the maximum concentration (C_{max}) level of levofloxacin, which can detect after aerosol injection [17]. Conversely, the oral administration of antibiotic drugs provides a level of concentration for both drugs in the range 10-20 μg/mL [47, 48], which is higher than the LOQs performed using this procedure.

The MEPS-HPLC-PDA apparatus allows to run samples under isocratic elution, thus deleting drawbacks, i.e. dead volumes, occurred using the same set up in different apparatus and equipments. The use of IS allows to monitor contineously the extraction and analysis of samples over the time. The MEPS-HPLC-PDA apparatus also requires short time (15 minutes) to complete analysis. The short time of analysis may provide several advantages in clinical studies.

In the reported procedure, MEPS extraction was performed in "*off-line*" mode, but this is not a limiting step, especially related to the HPLC isocratic mode separation (easy method transfer also to other HPLC instrument) and all MEPS procedures reported herein can be directly configured into the software system (for *on-line* measurements) without any loss of chromatographic resolution and/or analytical performances related to the autosampler void volume. The major advantage of the developed extraction procedure is that MEPS device, like SPE, is "*field portable*" for remote sampling without the use of automated equipment with the sample loading step (*field based*) and clean up/elution (*laboratory based*) separated, particularly adapted to small sample volume analysis. In *off-line* configuration, the manual operation of the syringe pump allowed sampling without the

441 need for portable power supplies or other sampling equipments. Additionally the use of *off-line* MEPS
442 configuration can reduce the need to recover and stabilize samples for transport to the laboratory, and
443 permit to use this device for *off-line* analysis by NMR, and IR methods including immunoassay. The
444 reported procedure allows also to eliminate all extra steps between sample preparation and sample
445 injection.Table 3 lists the extraction procedure, the instrument setting up, the elution mode, the
446 chromatographic analysis and the analytical parameters used to detect ciprofloxacin and/or
447 levofloxacin extracted from different biological samples.
448

449 **Table 3:** Analytical methods show the analysis of ciprofloxacin and/or levofloxacin extracted from different biological samples.

450

Sample	Analytes [a] (FLQs)	Extraction [b] (Requested time)	Instrument Setting up	Elution	LOD	LOQ	Chromatographic analysis (Minutes)	Year	Ref.
Plasma	Ciprofloxacin	PP (10 minutes)	HPLC-FLD	Gradient	Not reported	0.0412 μg/mL	16	2003	20
Plasma	Ciprofloxacin	SPE (Not reported)	HPLC-FLD	Gradient	Not reported	16.56 μg/mL	16	2005	21
AHH	Ciprofloxacin	PP/dilution (5 minutes)	HPLC-UV/Vis	Isocratic	0.004 μg/mL	0.008 μg/mL	10	2008	22
Plasma	Ciprofloxacin	PP (15 minutes)	HPLC-UV/Vis	Isocratic	0.083 μg/mL	0.169 μg/mL	15	2008	23
Serum Urine CSF Bronchial	Levofloxacin	PP/dilution (Not reported)	UHPLC-ESI-MS/MS	Gradient	0.04 μg/mL 0.1 μg/mL 0.016 μg/mL 0.008 mg/kg	0.1 μg/mL 0.5 μg/mL 0.053 μg/mL 0.027 mg/kg	6.0 (ES^+) and 5.8 (ES^-)	2014	25
Urine	Ciprofloxacin	Dilution (Not reported)	Fluorescent spectroscopy	-	Not reported	0.2 μg/mL	Not reported	2009	27
Plasma ***Sputum***	***Moxifloxacin***	***PP (Not reported)***	***HPLC-UV/Vis***	***Isocratic***	***Not reported***	0.078 μg/mL ***0.1563 μg/mL***	***Not reported***	***2009***	***29***
Sputum	***Ciprofloxacin***	***LLE (Not reported)***	***HPLC-FLD***	***Gradient***	***0.05 μg/mL***	***Not reported***	***Not reported***	***1987***	***30***
Compost	Levofloxacin Ciprofloxacin	MAE (20 minutes)	UPLC-HESI-MS	Isocratic	2.9 ng/g 3.0 ng/g	8.6 ng/g 9.0 ng/g	14	2015	41
Plasma	Levofloxacin	LLE (10 minutes)	HPLC-FLD	Gradient	0.01 μg/mL	0.05 μg/mL	25	2006	42
Urine	Ciprofloxacin	MIP (Not reported)	HPLC-FLD	Gradient	0.0049 μg/mL	0.016 μg/mL	25	2009	43

451

Table 3 cont.: Analytical methods show the analysis of ciprofloxacin and/or levofloxacin extracted from different biological samples.

Sample	Analytes [a] (FLQs)	Extraction [b] (Requested time)	Instrument Setting up	Elution	LOD	LOQ	Chromatographic analysis (Minutes)	Year	Ref.
Serum	Ciprofloxacin	PP/LLE/evaporation (Not reported)	HPLC-FLD	Isocratic	0.06 μg/mL	0.125 μg/mL	10	1986	44
Broth	Levofloxacin	PP (Not reported)	HPLC-FLD	Isocratic	Not reported	0.15 μg/mL	Not reported	2013	45
Urine	Levofloxacin Ciprofloxacin	Dilution/Precipitate (Not reported)	UV and AAS	-	0.4-0.5 μg/mL 0.4-0.46 μg/mL	Not reported	Not reported	2005	46
Sputum	***Levofloxacin Ciprofloxacin***	***MEPS (10 minutes)***	***HPLC-PDA***	***Isocratic***	***0.017 μg/mL***	***0.05 μg/mL***	***15***	***Current paper***	***Current paper***

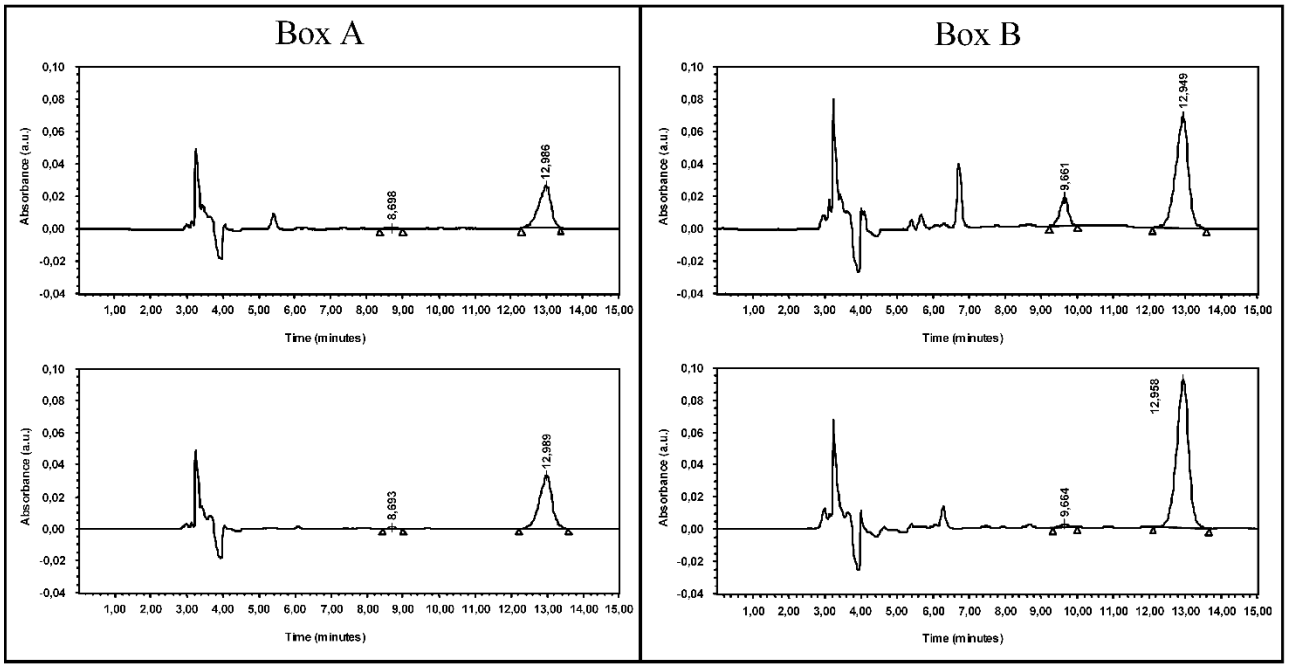
[a] fluoroquinolones detected using procedure for the simoultaneous analysis of different drugs; [b] extraction time depends on the centrifugation time; AHH = aqueous human humor; PP = protein precipitations; LLE = liquid-liquid extraction; MAE = microwave assisted extraction; MIP = molecularly imprinted polymer; CSF = cerebrospinal fluid; ES^+ = positive ionisation mode; ES^- = negative ionisation mode; FLD = fluorescence detection; HESI = Heated Electro Spray Ionisation; AAS = atomic absorption spectrometric.

At the best of our knowledge, there is no MEPS-HPLC-PDA procedure in liturature, which allows to detect simultaneously, and regardless of clinical therapy, ciprofloxacin and levofloxacin collected from sputum of patients affected from CF with comparable performances. Furthermore, the MEPS-HPLC-PDA apparatus shows great analytical performance in biological samples compared to other expensive and complex analytical procedures.

3.5 Application to real CF sputum samples

The resultant method was tested to quantify ciprofloxacin and levofloxacin in sputum samples collected from CF patients after IV or per OS administration. The collected samples were extracted using MEPS system and analyzed by HPLC-PDA apparatus. Figure 3 shows chromatograms obtained after the analysis of real samples (see also Supplementary Material, Section S.6 for all chromatograms at maximum wavelengths).

Figure 3. Chromatograms obtained after the analysis of real samples. Drugs: Levofloxacin (IV top, OS bottom) box A at 295 nm, and Ciprofloxacin (IV top, OS bottom) box B at 279 nm; injected dose: 500 mg × 2/die.



The quantitative analysis of real samples were further showed in Table 4.

Table 4: Quantitative analysis of sputum samples collected from patients treated with Levofloxacin and Ciprofloxacin.

Sample #	Way of Administration	Drug Treatment	Ciprofloxacin (μg/mL)	Levofloxacin (μg/mL)	Time (hours) [a]	Sample Volume (mL)
1	OS	Levofloxacin (500 mg × 2/die)	-	0.21	2	2
2	IV	Levofloxacin (500 mg × 2/die)	-	BLQ	2	2
3	OS	Ciprofloxacin (500 mg × 2/die)	0.10	-	2	2
4	IV	Ciprofloxacin (500 mg × 2/die)	1.06	-	8	2
5	CTRL	-	-	-	-	2
6	CTRL	-	-	-	-	2

OS: oral administration; IV: intravenous administration: CTRL: control. [a]Time between last fluoroquinolone administration and sputum collection.

Sputum samples were collected 2 hours after the last fluoroquinolone administration for samples 1, 2, and 3, and after 8 hours for sample 4. Low CF sputum concentration levels for levofloxacin (0.21 μg/mL) and ciprofloxacin (0.10 μg/mL) after 2 hours of injection per OS administration depend on the low serum C_{max} (approximatly 7 μg/mL) level at t_{max} (2.2 hours) as reported by Lee and coworkers [49]. Additionally, the high ciprofloxacin concentration after IV administration agreed published data of Payen and coworkers [50].

CF patients are highly susceptible to bacterial respiratory infections. Therefore, they underwent to repeated, intensive and prolonged cycles of antibiotic therapy to maintain the lung function and to reduce the number of pulmonary exacerbations. Treatment of pulmonary infectious in CF patients is a challenge for the clinician due to the multi-drug resistance phenotype of bacterial pathogens and to the unpredictable pharmacokinetic alterations arising from the complex pathophysiological changes observed in this population. To optimise the efficacy of the antibiotic treatment of lung infections in CF patients, the current strategies recommend intravenously administered ciprofloxacin at 20-30 mg/Kg/day [51].

As previously reported in literature, the mean ciprofloxacin concentration in sputum determined at 1, 2, or 4 h after OS and IV administration in CF patients ranged from 0.16 to 1.64 and from 1.02 to 0.39 μg/mL, respectively [52]. At the best of our knowledge, and as additional novelty of the reported work, no similar data are available for levofloxacin following OS or IV administration and determined in sputum samples. This is another novelty of the herein reported work, that permit the evaluation of the antibiotic treatment efficacy when different clinical protocol were adopted.

4. Conclusions

The determination and quantification of levofloxacin and ciprofloxacin using MEPS-HPLC-PDA in sputum samples collected from CF patients were successfully performed through a Discovery C_8 column using a binary solvent system (86:14, *v:v*) made from phosphate buffer (30 mM, pH 2.5, 1% TEA), and AcN (1% TEA) at 1.0 mL/min flow rate.

The analytical performance was validated and the method was successfully tested to quantify the ciprofloxacin and levofloxacin in sputum samples collected from CF patients, which were injected per OS or IV under antibiotic therapy. In the explored range the method is accurate, selective, and sensitive enough to allow the analysis of antibiotic drugs in sputum after MEPS extraction. Neither endogenous compounds nor other co-administered drugs showed significant interferences in terms of selectivity. The analyses can be carried out by means of a relatively simple procedure, with a decrease of analytical variability and sample handling time. These advantages depend also on the use of IS, and the isocratic elution mode. Our results further suggest that MEPS-HPLC-PDA can be a suitable tool to detect, separate and quantify efficiently the levofloxacin and ciprofloxacin from biological samples, and can represent an innovative therapeutic strategy for the analysis of antibiotic drugs in clinic. Another main advantage is represent by the simultaneously and regardless of clinical therapy quantification of these two drugs in sputum collected from CF patients. The MEPS-HPLC-PDA in *off-line* mode can also represent an easy, fast, routinary and cheap analytical method to various antibiotic drugs in biological samples without using time consuming and expensive apparatus, which require specialists and optional configurations.

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Figure and Table captions

Table 1: Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent eight non-zero concentration points.

Table 2: Intra-day and Inter-day precision (RSD%), trueness (Bias%) of the analytical method obtained from the analysis of QC samples.

Table 3: Analytical methods show the analysis of ciprofloxacin and/or levofloxacin extracted from different biological samples.

Table 4: Quantitative analysis of sputum samples collected from patients treated with Levofloxacin and Ciprofloxacin.

Figure 1. Chemical structures of Ciprofloxacin, Levofloxacin, and Enrofloxacin (IS).

Figure 2. Chromatograms obtained after the extraction and analysis of ciprofloxacin, levofloxacin, and enrofloxacin at 279, 295, and 278 nm, respectively (trace a: blank human sputum, trace b: blank human sputum spiked with 1 μg/mL of Internal Standard, and trace c: blank human sputum spiked with 1 μg/mL of Internal Standard and 0.75 μg/mL of analytes). 20 μL of samples was injected during the analysis.

Figure 3. Chromatograms obtained after the analysis of real samples. Drugs: Levofloxacin (IV top, OS bottom) box A at 295 nm, and Ciprofloxacin (IV top, OS bottom) box B at 279 nm; injected dose: 500 mg × 2/die.