Development and validation of a UPLC-MS/MS method for simultaneous quantification of levofloxacin, ciprofloxacin, moxifloxacin and rifampicin in human plasma: Application to the therapeutic drug monitoring in osteoarticular infections

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Abstract

BACKGROUND: Fluoroquinolones and rifampicin are antibiotics frequently used for the treatment of osteoarticular infections, and their therapeutic drug monitoring is recommended. The aim of this study was to develop and validate a rapid and selective method of simultaneous quantification of levofloxacin, ciprofloxacin, moxifloxacin and rifampicin with short pretreatment and run times in order to be easily used in clinical practice.

METHODS: After a simple protein precipitation of plasma samples, the chromatographic separation was performed using an ultra-performance liquid chromatography system coupled with mass tandem spectrometry in a positive ionization mode. The mobile phase consisted of a gradient elution of water-formic acid (100:0.1, v/v)-ammonium acetate 2 mM (A) and methanol-formic acid (100:0.1, v/v)-ammonium acetate 2 mM (B) at a flow rate at 0.3 mL/min.

RESULTS: Analysis time was 5 minutes per run, and all analytes and internal standards eluted within 0.85-1.69 minutes. The calibration curves were linear over the range from 0.5 to 30 µg/mL for levofloxacin, ciprofloxacin, moxifloxacin and rifampicin with linear regression coefficients above 0.995 for all analytes. The intra-day and inter-day coefficients of variation were below 10% for lower and higher concentration. This method was successfully applied to drug monitoring in patients with an osteoarticular infection.

CONCLUSION: A simple, rapid, and selective liquid chromatography-tandem mass spectrometry method was developed and validated for the simultaneous quantification of levofloxacin, ciprofloxacin, moxifloxacin and rifampicin in human plasma.

Keywords: liquid chromatography, mass spectrometry, therapeutic drug monitoring, antibiotics, osteoarticular infections.

1. Introduction

Osteoarticular infections (OAIs) are a fairly common type of infection with an incidence of 54.6 cases per 10,000 persons in France [1]. OAIs have variable expressions depending on the context and age of patients. However, management follows common principles: identification of the infectious agent, mapping of osteoarticular involvement and implementation of prolonged antibiotic therapy [2]. The choice of antibiotic therapy is essentially based on the ability of antibiotics to distribute to the infected site. The use of antibiotics with good bone penetration therefore is a priority. Fluoroquinolones (FQs) such as levofloxacin (LVX), ciprofloxacin (CPX), moxifloxacin (MOX) and rifampicin (RIF) member of the rifamycin agents, show very good bone diffusion [3] and are frequently used in this context. Moreover, given the high risk of resistant mutant selection when using these antibiotics as monotherapy, and in order to ensure the widest possible coverage spectrum, both FQs and RIF should always be used in combination in another antibiotic. The association between FQs and RIF is very common in OAIs, especially during infection due to Gram positive bacteria, hence the relevance of the ability to measure them simultaneously in patient plasma. In addition, given their high intra- and inter-individual pharmacokinetic (PK) variability, the risk of drug interaction and the need to limit the occurrence of resistance due to improper dosing, therapeutic drug monitoring (TDM) of these antibiotics is highly recommended [4,5]. FQs and RIF are concentration-dependent bactericidal antibiotics [6]; their TDM is primarily based on the determination of their area under the curve (AUC). The $AUC_{0\text{-}24}$ / minimum inhibitory concentration (MIC) ratio for FQs and $AUC_{0\text{-}12}$ for RIF are the major parameters for predicting the clinical and microbiological efficacy of these antibiotics. The recommended therapeutic efficacy threshold for FQs is defined in the literature as a $AUC_{0\text{-}24}$ / MIC ratio > 125 for Gram negative bacteria, and > 35 for Gram positive bacteria [7–9]. The recommended therapeutic range for RIF corresponds to an $AUC_{0\text{-}12}$ between 30 and 65 $\mu g.h^{-1}.mL^{-1}$, and a

C_{max} between 8 and 24 µg/mL [10,11]. Several analytical methods such as liquid chromatography coupled to ultraviolet (LC-UV) [12,13] or mass spectrometry (LC-MS/MS) [14–19] are reported for the analysis of LVX, CPX, MOX and RIF alone or in combination with other compounds; yet, the established methods suffer in some cases from the limited range of covered concentrations and/or from a long run time, which make them less suitable in clinical practice. The aim of this study was to establish a rapid, selective and simple LC-MS/MS method for simultaneous analysis of LVX, CPX, MOX and RIF suitable for routine analysis and pharmacokinetic studies.

2. Materials and methods

2.1. Chemical and reagents

Levofloxacin (LVX), ciprofloxacin (CPX), moxifloxacin (MOX) and rifampicin (RIF) were purchased from Sigma-Aldrich (Munich, Germany). Levofloxacin-$^{2}H_{8}$ (LVX-d8) and ciprofloxacin-$^{2}H_{8}$ hydrochloride (CPX-d8) were purchased from LGC (Augsburg, Germany) while moxifloxacin-$^{2}H_{5}$ trifluoroacetate (MOX-d5) and rifampicin-$^{2}H_{8}$ (RIF-d8) were purchased from Alsachim (Illkrich, France). Methanol was obtained from Merck (Darmstadt, Germany). Ascorbic acid, formic acid and ammonium acetate were obtained from Sigma-Aldrich (Munich, Germany). Zinc sulphate heptahydrate was obtained from VWR (Fontenay-sous-Bois, France). All reagents used were of the highest available analytical grades. Liquid chromatography–MS/MS grade water was purchased from a water distribution hypergrade system Purelab Flex® (ELGA®), and blank human plasma was from the French Blood Establishment (Paris, France).

2.2. Calibration and quality control sample preparation

Stock solutions of each of the four antibiotics were prepared at 1 mg/mL. LVX, CPX and MOX were prepared in hydrochloric acid 0.2 M while RIF was prepared in methanol.

Calibration range and quality control (QC) samples were prepared in blank human plasma by adding the appropriate amount of working solutions of FQs mix and RIF. For LVX, CPX, MOX and RIF, calibration range concentrations were 0.5, 1, 5, 10, 20 and 30 µg/mL, respectively, and QC levels were 1, 10 and 30 µg/mL. A solution of mix of internal standard (IS mix) at 100 ng/mL for LVX-d8, and at 500 ng/mL for CPX-d8, MOX-d5 and RIF-d8 was prepared in methanol. All prepared solutions, calibration range and QC samples were stored at -20°C. RIF solutions were stabilized by supplementation with an adequate volume of ascorbic acid solution (pH 2.75; 0.11M) [20].

2.3. Instruments and analytical conditions

Chromatography was performed on an Acquity UPLC® system (WATERS®, Milford, Massachusetts, United States) with an autosampler temperature at +4°C. Acquity UPLC® BEH C18 column (4.6 x 150 mm, 3.5 µm particle size) was used for chromatographic separation and column temperature was maintained at 45°C. The mobile phase had a flow rate of 0.3 mL/min with a non-linear gradient elution and the run time analysis at 5 min. The UPLC system was coupled to a triple quadripole mass spectrometer: TQD Xevo® (WATERS®, Milford, Massachusetts, United States). Quantifications were achieved in Multiple Reactions Monitoring (MRM) mode and electrospray ionization (ESI) was operated in positive mode. The MS/MS instrument was set with capillary voltage (3.5 kV) and desolvation gas (nitrogen) heated at 380°C. Data acquisition was performed using MassLynx® 4.2 software.

2.4. Samples pre-treatment

Samples were prepared by adding 150 µL of $ZnSO_4*7H_2O$ solution (pH 5.40; 0.10 M), 300 µL of IS mix solution and 300 µL of ultrapure water to 50 µL of plasma sample, calibrator or QC. The mixture was then vortexed for 1 min and centrifuged for 10 min at 18,900 g at room

temperature. Finally, 5 µL of supernatant were injected into the LC-MS/MS system using a temperature-controlled autosampler device (+4°C).

2.5. Validation

The validation was performed according to European Medicines Agency (EMA) guidelines and US Food and Drug Administration (FDA) guidelines for the validation of bioanalytical methods. Parameters included were selectivity, linearity, accuracy and precision, lower limit of quantification, matrix effect, stability in human plasma and dilution integrity.

2.5.1. Selectivity

Six different sources of plasma samples were tested. A selective method should not have interference of more than 20% of the lower limit of quantification (LLOQ) of the analyte.

2.5.2. Carry-over

Carry-over was assessed by injecting blank samples after a high concentration calibrator. Carry-over in the blank sample following the high concentration calibrator should not be greater than 20% of the LLOQ.

2.5.3. Linearity

Calibration curves were acquired by plotting the peak area ratio of the concentration of each LVX, CPX, MOX and RIF standard to the area of their respective IS over the range from 0.5 to 30 µg/mL. Each curve was assayed by least square weighted (1/x). Linearity was defined by a linear regression coefficient $r^2 \geq 0.995$.

2.5.4. Precision and accuracy

The intra-day assay precision and accuracy were evaluated using 30 replicates of QC samples at the three concentration levels (1, 10 and 30 µg/mL) for LVX, CPX, MOX and RIF. The

concentrations of controls were chosen to cover the range of the calibration curve.

The inter-day assay was determined by repeating each QC sample twice a day over 15 different days. The concentration of each sample was determined using calibration standards prepared on the same day. The precision was calculated as the coefficient of variation (CV, %) within a single run (intra-day assay) and between different assays (inter-day assay) and the accuracy as the bias between nominal and measured concentration. The acceptance limits were CV < 15% for precision and within ± 15% of the nominal concentration for accuracy.

2.5.5. Lower limit of quantification

To determine the LLOQ of LVX, CPX, MOX and RIF, serial dilutions in blank human plasma, of the lowest point of the calibration range (0.5 µg/mL) were prepared to obtain 9 samples at the respective concentration of 0.45, 0.40, 0.35, 0.30, 0.25, 0.20, 0.15, 0.10 and 0.05 µg/mL. The precision and accuracy were evaluated using 10 replicates of each sample on the same day (intra-day assay), and for 3 consecutive days (inter-day assay; n = 30). The concentration of each sample was determined using calibration standards prepared on the same day. The LLOQ corresponds to the lowest concentration sample with a CV < 20% and within ± 20% of the nominal concentration.

2.5.6. Matrix effect and extraction recovery

Matrix effect (ME) analysis was performed to determine the possible ionization enhancement or suppression by sample matrices. The approach involves determination of ratio of peak areas of analyte in three different sets, one consisting of aqueous standards (set A), one prepared in blank matrix extracts and spiked after extraction (set B), and one prepared in blank matrix from the same sources but spiked before extraction (set C). ME and extraction recovery (ER) were calculated by the following equations: ME (%) = B/A*100 and ER (%) = C/B*100. It was assessed at the three levels of QC (1, 10 and 30 µg/mL) in quintuplicate. A

value above or below 100% for the ME indicates an ionization enhancement or suppression respectively. ME was considered negligible for a ratio ranged from 85% to 115%.

2.5.7. Stability

Stability in plasma of LVX, CPX, MOX and RIF was tested by comparing the observed bias between baseline concentration of three QC samples (1, 10 and 30 µg/mL) and the mean concentration obtained after different storage conditions using freshly prepared calibrators. The stability of the analytes in the plasma was tested immediately after sample preparation (baseline) and after storing them at room temperature, +4°C and -20°C. Concentration of each analyte was then determined after 24, 48 and 72 hours, 1 and 2 weeks and 1 and 3 months. The stability after three freeze and thaw cycles was also tested. For this purpose, samples stored for a minimum of 12 hours at -20°C, were kept at room temperature for at least 30 minutes followed by freezing in -20°C for a minimum of 12 hours.

All stability tests were done in triplicate per QC level. For each molecule, it was considered to be stable in plasma sample when mean measured concentrations within ± 15% of the baseline concentration.

2.5.8. Dilution integrity

The dilution integrity was examined to ascertain that an unknown sample with concentration exceeding the upper limit of compounds calibration range, could be diluted with blank matrix without influencing the accuracy and precision of the measurement. To achieve this, a sample was prepared at higher concentration (40 µg/mL) followed by dilution (1:1) in blank plasma before extraction. Diluted sample was done in quintuplicate. The inaccuracy and imprecision of the diluted sample should not deviate by more than 15%.

2.6. Clinical application

Blood samples were obtained in patients with an osteoarticular infection hospitalized in the orthopedic surgery department at Pitié-Salpêtrière who were treated, by oral administration, with LVX or CPX or MOX and / or RIF. Blood samples were collected into heparin tubes before dosing (t_0) and 1, 2 and 5 hours post-dosing at steady state. The median time of blood collection was 5 (range: 3-12) days after start of treatment. AUC were performed using WinNonLin® 4.1 software. AUC_{0-12} and AUC_{0-24} were estimated considering that, at steady state, the 12 or 24 hours post-dosing concentration was equal to the before dosing concentration (t_0). Plasma samples were prepared by centrifuging collected blood samples for 5 min at 4,500 g at room temperature. All plasma samples were frozen at -20°C until analysis. French regulations on non-interventional observational studies do not require patient's consent when analyzing data obtained from routine care. Approval for data collection was obtained from the Commission Nationale de l'Informatique et des Libertés (n°1491960v0).

3. Results

3.1. Optimization of LC-MS/MS conditions

Electrospray positive mode yielded a better spectrometer response than the negative mode. To achieve symmetrical peak shapes, good resolution and a short chromatographic run time, a mobile phase consisting of (A) water-formic acid (100:0.1, v/v)-ammonium acetate 2 mM (pH* 2.82) and (B) methanol-formic acid (100:0.1, v/v)-ammonium acetate 2 mM (pH* 4.30) was used in the experiments. Mass spectrometry parameters for the LC-MS/MS determination of LVX, CPX, MOX, RIF and their respective IS are shown in Table 1.

3.2. Method validation

3.2.1. Selectivity and carry-over

Six plasma samples without LVX, CPX, MOX and RIF but containing the following drugs: topiramate, diazepam, lansoprazole, levetiracetam, clonazepam, paracetamol, furosemide,

229 lacosamide and amoxicillin were tested. No interference with endogenous compounds or
230 tested drugs was observed above 20% of the LLOQ of the analytes and with the same
231 transitions and retention times of the studied analytes or their respective IS (Figure 1.A, 1.B).
232 The retention time of LVX, CPX, MOX and RIF was 0.86, 1.02, 1.34 and 1.69 min
233 respectively. The carry-over observed with the different analytes was less than 20% of the
234 LLOQ [LVX (3%), CPX (8%), MOX (11%) and RIF (4%)]. Furthermore, no carry over was
235 observed for any of the IS used.

236 3.2.2. Linearity

237 Calibration curves were linear with linear regression coefficient greater than $r^2 = 0.9994$ for
238 all analytes (from 0.9994 to 0.9998). All calibrators, analyzed on 15 different days, were
239 measured with an inaccuracy ranged from -3.5% to 6.5% and an imprecision of less than
240 3.7%. The highest calibration point (30 µg/mL) was defined as upper limit of quantification
241 (ULOQ) for all analytes. The linear regression equations were $y = 1.42712x + 0.05266$; $y =$
242 $0.51421x + 0.01040$; $y = 0.54308x + 0.04832$ and $y = 0.32515x + -0.02830$ for LVX, CPX,
243 MOX and RIF respectively.

244 3.2.3. Accuracy and precision

245 Intra- and inter-day precision and accuracy outcomes of QC samples are shown in Table 2.
246 The intra- and inter-day inaccuracy ranged from -10% to -0.5% and from -4.1% to 5.3%
247 respectively, for all analytes at all tested concentrations. Likwise, the intra- and inter-day
248 imprecision were less than 1.3% and 5.6%, respectively, for all the analytes at all tested
249 concentrations.

250 3.2.4. Lower limit of quantification

The LLOQ was established at 0.25 µg/mL for LVX; 0.15 µg/mL for CPX; 0.10 µg/mL for MOX and 0.30 µg/mL for RIF. The chromatogram of the different analytes at their LLOQ is shown in Figure 1.A.

3.2.5. Matrix effect and extraction recovery

Matrix effect and extraction recovery for all the analytes ranged from 93.4% to 108.3% and 91.2% to 105.7%, respectively, and were constant over the concentration range for each of them, as shown in Table 3. The result of ME indicated that there was no significant ionization suppression or maximization resulting from sample matrices. Moreover, the method resulted in high recovery value at all QCs showing good efficiency.

3.2.6. Stability

Table 4 shows the stability in plasma. The data presented correspond to the bias between the mean measured concentration and the baseline concentration of LVX, CPX, MOX and RIF using different test conditions. LVX and MOX remained stable for 3 months regardless of the storage temperature. CPX remained stable for 3 months at -20°C; 1 month at room temperature and +4°C. Stabilized RIF (with ascorbic acid) remained stable for 3 months at -20°C; 1 month at +4°C and only 48 hours at room temperature.

Regarding freeze and thaw stability, QC samples were stable after three freeze and thaw cycles. All samples were measured with inaccuracy (bias from baseline concentration, %) ranging from -1.3% to -6.1% for all analytes at all tested concentrations and with imprecision (CV, %) ranging from 2.1% to 4.5% for all analytes at all tested concentrations.

3.2.7. Dilution integrity

The inaccuracy (bias, %) and imprecision (CV, %) of the diluted samples were: LVX (-1.4; 2.9), CPX (-3.9; 3.1), MOX (-0.4; 4.3) and RIF (8.7; 2.8).

3.3. Clinical application

This validated LC-MS/MS method was successfully applied to the TDM of twenty seven patients with an osteoarticular infection treated with LVX 500 mg BID (n=6) or CPX 750 mg BID (n=6) or MOX 400 mg QD (n=3) or RIF 600 mg BID (n=12). The chromatograms of RIF at 1.4 µg/mL, LVX at 3.2 µg/mL, CPX at 0.4 µg/mL and MOX at 1.2 µg/mL of patients at t_0 are shown in Figure 2.C. The mean age of patients was 57 ± 19 years old with sex ratio of 1.9. All patients had normal kidney and liver function. A representative concentration versus time profile of RIF, LVX, CPX and MOX is shown in Figure 2, and their pharmacokinetic parameters are shown in Table 5.

4. Discussion

The newly developed and validated method allows accurate and fast quantification of four antibiotics frequently used in the treatment of osteoarticular infections. Only one currently published method combine the simultaneous analysis of LVX, CPX, MOX and RIF [17] even though the association between FQs and RIF is commonly used. Being able to quantify these two classes of antibiotics by the same technique is therefore a major advantage. One of the difficulties of the simultaneous analysis of these four antibiotics lies in the difference in the expected plasma concentration of each of these antibiotics. To simplify routine execution, we chose to perform a calibration range with an identical concentration range for the four antibiotics. This new method has a high concentration range (from 0.5 to 30 µg/mL) which makes it suitable for the measurements of the maximum and minimum concentration (C_{max}, C_{min}) reported for LVX, CPX, MOX and RIF in plasma. In comparison to other methods, the current one has an extended upper limit for all four antibiotics, even though lower quantification limits are attained by other methods. Kim et al. have developed a method for

quantification of 20 anti-tuberculosis drugs including LVX, CPX, MOX and RIF [17] with a calibration range from 0.2 to 10 µg/mL for these four antibiotics. Likewise, Jourdil et al. have also developed a method for quantification of 9 antifungals and antibiotics drugs including LVX, CPX and RIF [18] with a calibration range from 0.04 to 6 µg/mL for LVX and CPX, and from 0.2 to 8 µg/mL for RIF, respectively. These two methods might be not suitable for the measurements of the C_{max} reported for RIF and LVX in clinical practice. In addition, in our method, in case of concentrations exceeding our ULOQ, especially for LVX and RIF, the dilution integrity test shows that the sample can be diluted in blank plasma without affecting analysis quality.

Concerning the sample preparation and run time analysis, in the method developed by Kim et al. [17], the sample pretreatment was similar to ours (protein precipitation with methanol containing ISs) but the run time was 9 min. Our method is faster (run time of 5 min) which could reduce the time required for quantification of large number of samples. In the method developed by Jourdil et al. [18], the run time was 4 min but, MOX was not quantified. Furthermore, with respect to other methods, our method is the only one in which LVX, CPX, MOX and RIF are analyzed with respect to their respective isotopically internal standard.

Regarding stability, we performed a 3-month stability study. Lee et al. showed in their study that LVX and MOX were stable for 3 months at -80°C in human serum [19]. We have shown in our study that this was also the case at -20°C in human plasma. Furthermore, to our knowledge, our study is the first to demonstrate the stability of RIF stabilized by ascorbic acid for 3 months at -20°C in human plasma. In their study, Kim et al. [17] showed a degradation of RIF (studied without the addition of ascorbic acid) in plasma after storage at -80°C for 3 months. Our study shows that the addition of ascorbic acid greatly improves the stability of RIF in plasma. These stability data are important information for the storage of patient plasma

samples. This is particularly important in the case of clinical research protocols, where samples may need to be stored for a long time before they can be assayed.

Concerning the clinical application, 3 of 12 patients taking RIF had a C_{max} and an AUC_{0-12} below the expected minimum values of 8 µg/mL and 30 $µg.h^{-1}.mL^{-1}$, respectively, and 4 of 12 patients had an AUC_{0-12} above the expected maximum values of 65 $µg.h^{-1}.mL^{-1}$. Moreover, as shown in Table 5 and Figure 2, there is a very large inter-individual pharmacokinetic variability of FQs and RIF in patients treated for osteoarticular infection. In this context, TDM could be a powerful technique to measure and adjust the dose, which is especially important for concentration-dependent bactericid antibiotics such as FQs and RIF. Using this method, 8 out of 27 patients were actually able to benefit from a dose adjustment based on their pharmacokinetic profiles. In the end, the method developed could be used in a PK/PD study to support the relevance of TDM in osteoarticular infections.

5. Conclusion

We have developed and validated a rapid, selective, simple, accurate, precise and reliable LC-MS/MS method for the simultaneous quantification of levofloxacin, ciprofloxacin, moxifloxacin and rifampicin in human plasma. This technique is currently used in clinical practice, particularly for drug monitoring in the treatment of osteoarticular infection.

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Figure 1.A Chromatograms **at LLOQ** of (A) LVX, (B) LVX-d8, (C) CPX, (D) CPX-d8, (E) MOX, (F) MOX-d5, (G) RIF, (H) RIF-d8

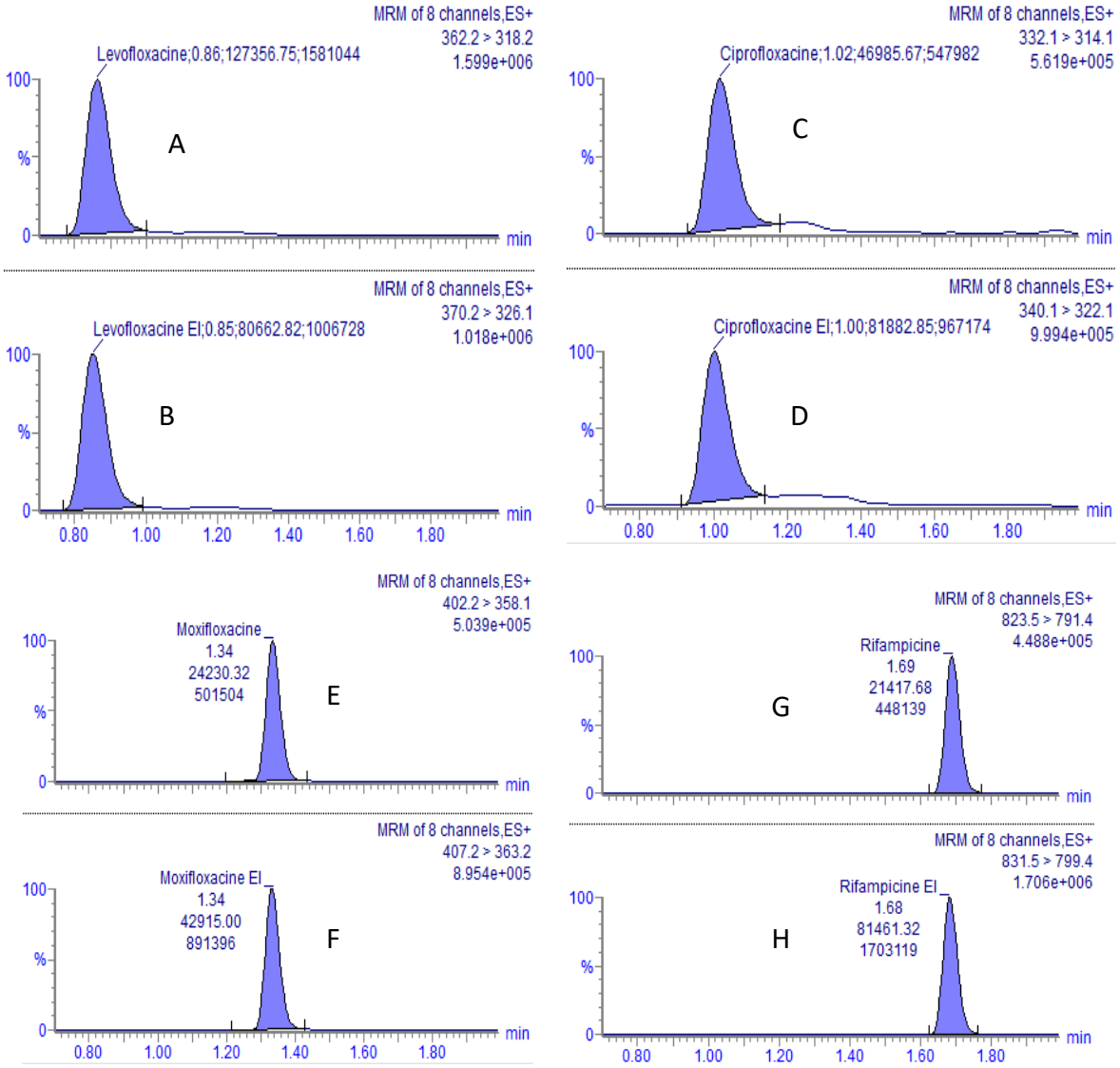


Figure 1.B blanks plasma of (A) LVX, (B) LVX-d8, (C) CPX, (D) CPX-d8, (E) MOX, (F) MOX-d5, (G) RIF, (H) RIF-d8

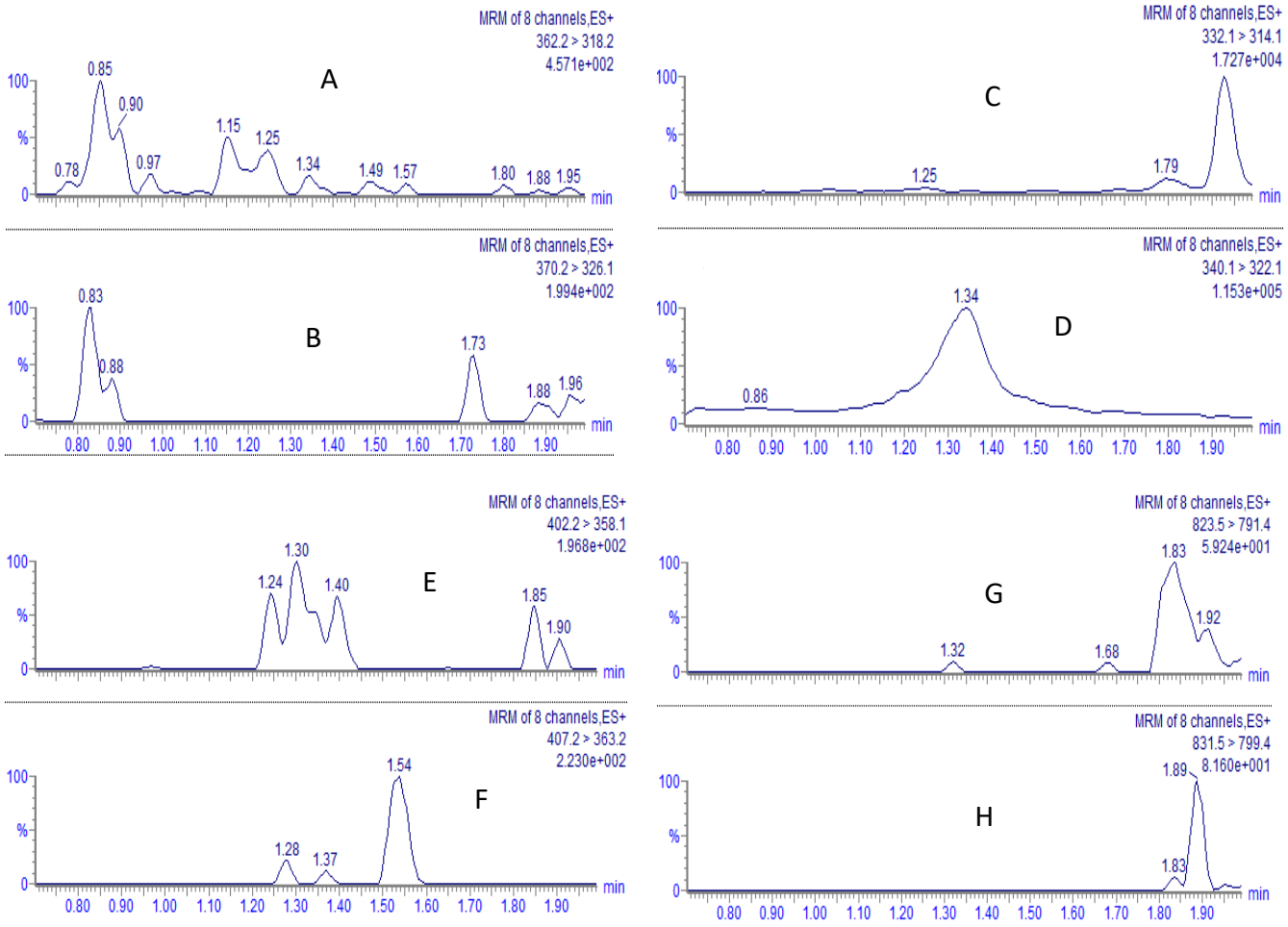
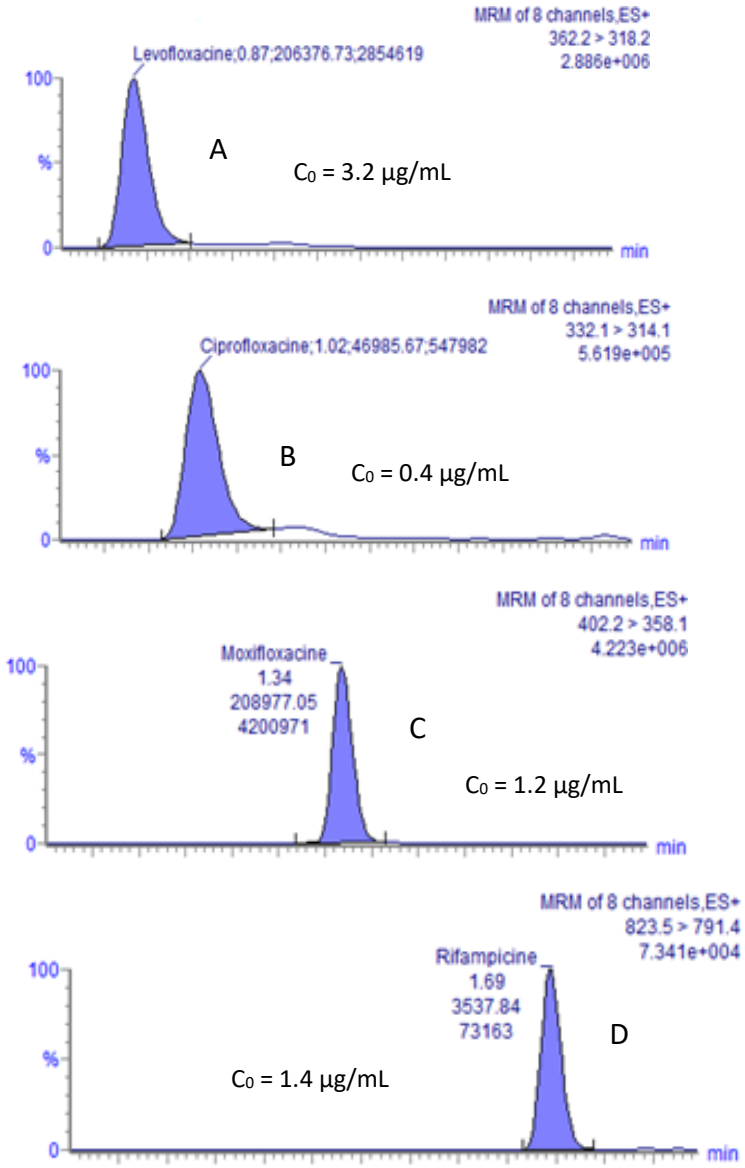


Figure 1.C Chromatograms of patients sample at t_0 of (A) LVX, (B) CPX, (C) MOX, (D) RIF.



C_0 : Concentration of patient sample at t_o

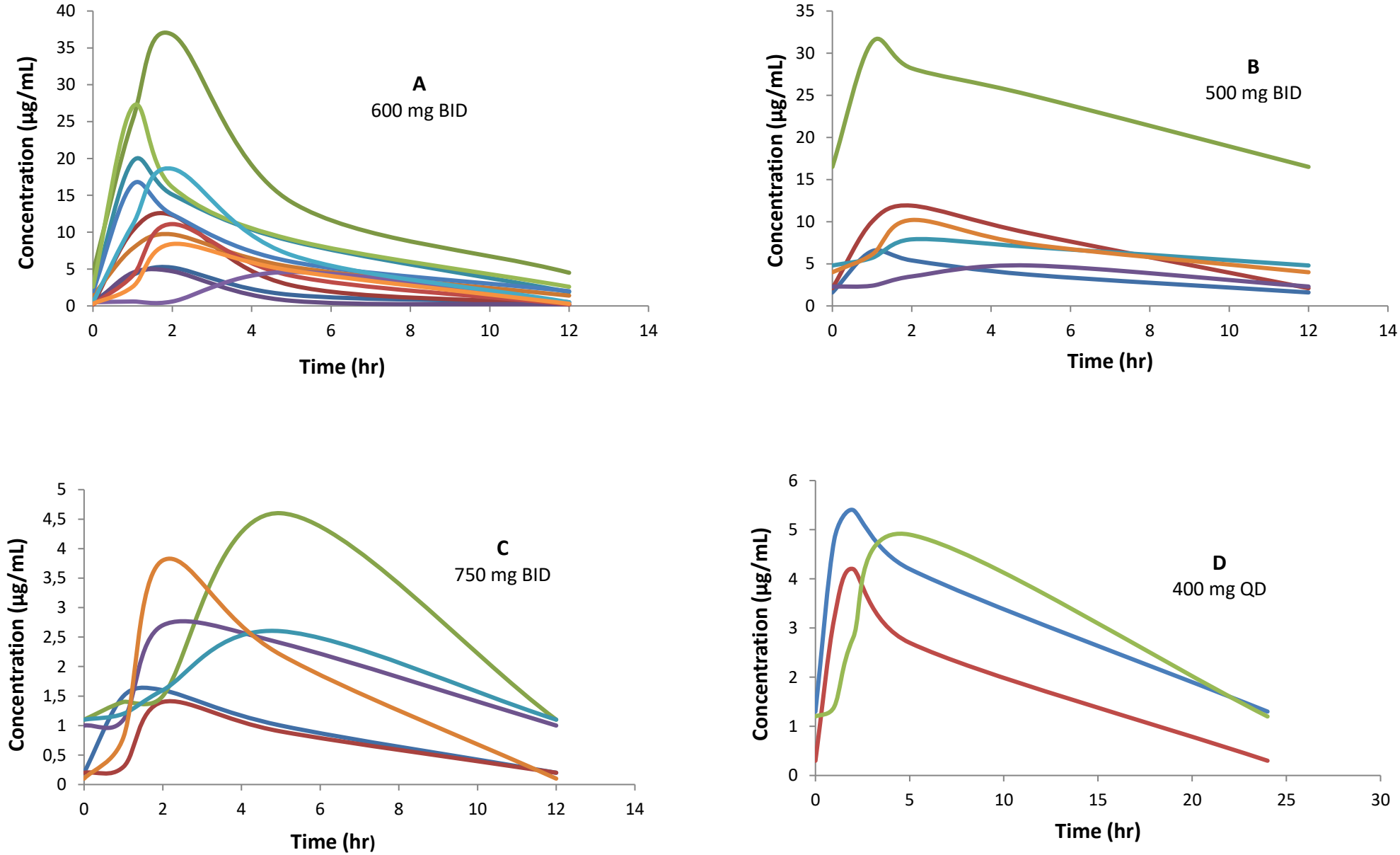


Figure 2. Concentration versus time profile of (A) rifampicin (N=12), (B) levofloxacin (N=6), (C) ciprofloxacin (N=6) and (D) moxifloxacin (N=3)

Table 1. LC-MS/MS parameters for the analysis of levofloxacin, ciprofloxacin, moxifloxacin, rifampicin and internal standards.

Analyte	Retention time (min)	MRM-Transtions (m/z)	collision energie (V)	cone potential (V)	Dwell time (ms)
LVX	0,86	362,2 > 318,2	18	40	38
LVX-d8	0,85	370,2 > 326,1	18	40	38
CPX	1,02	332,1 > 314,1	20	35	38
CPX-d8	1,00	340,1 > 322,1	20	40	38
MOX	1,34	402,2 > 358,1	20	35	38
MOX-d5	1,34	407,2 > 363,2	20	35	38
RIF	1,69	823,5 > 791,4	18	40	38
RIF-d8	1,68	831,5 > 799,4	18	35	38

Table 2. intra- and inter-day accuracy and precision of LVX, CPX, MOX and RIF

		Intra-day assay (n=30)				Inter-day assay (2/day; 15 days : n=30)			
	NC (µg/mL)	Mean measured concentration (µg/mL)	SD	Imprecision (%)	Inaccuracy (%)	Mean measured concentration (µg/mL)	SD	Imprecision (%)	Inaccuracy (%)
	1	0,96	0,01	0,8	-3,6	0,99	0,03	2,9	-1,5
LVX	10	9,8	0,1	0,5	-1,9	9,9	0,4	3,6	-0,8
	30	28,3	0,2	0,8	-5,8	28,8	1,2	4,1	-4,1
	1	0,98	0,01	1,1	-2,0	0,97	0,04	4,6	-3,2
CPX	10	10,0	0,1	0,7	-0,5	10,1	0,6	5,6	1,4
	30	28,5	0,2	0,8	-5,1	29,5	1,2	4,1	-1,5
	1	0,96	0,01	1,1	-4,1	1,05	0,06	5,4	5,3
MOX	10	9,7	0,1	0,8	-3,1	10,1	0,5	4,9	1,3
	30	28,6	0,2	0,8	-4,6	30,1	1,4	4,7	0,2
	1	0,90	0,01	1,0	-10,0	0,99	0,03	3,3	-0,7
RIF	10	9,2	0,1	0,9	-8,0	9,7	0,4	4,1	-3,3
	30	27,2	0,4	1,3	-9,3	29,6	1,5	5,1	-1,3

SD: standard deviation. NC: nominal concentration. CV: coefficient of variation.

Table 3. Matrix effect (ME) and Extraction recovery (ER) of 3 levels of controls: LOW (1µg/mL), MED (10µg/mL) and HIGH (30µg/mL)

	LOW		**MED**		**HIGH**	
n = 5	ME (%) + CV (%)	ER (%) + CV (%)	ME (%) + CV (%)	ER (%) + CV (%)	ME (%) + CV (%)	ER (%) + CV (%)
LVX	105.5 + 8.5	103.4 + 9.2	98.1 + 4.3	91.2 + 8.4	101.3 + 8.3	96.3 + 6.3
CPX	100.2 + 6.2	98.7 + 5.8	93.4 + 7.7	95.4 + 7.3	96.7 + 6.8	97.4 + 5.3
MOX	94.1 + 6.5	102.8 + 6.3	97.2 + 6.4	104.3 + 6.5	103.3 + 5.7	94.8 + 3.4
RIF	108.3 + 4.7	105.7 + 10.4	97.6 + 6.1	96.8 + 5.7	101.7 + 6.2	102.7 + 5.5

CV: coefficient of variation. n: number of replicates.

Table 4. Stability of LVX, CPX, MOX and RIF (stabilized by ascorbic acid) at room temperature, +4°C and -20°C. The data presented correspond to the bias between the mean measured concentration and the baseline concentration of 3 levels of controls: LOW (1 µg/mL), MED (10 µg/mL) and HIGH (30 µg/mL) in plasma.

	LVX			**CPX**			**MOX**			**RIF**		
n = 3	LOW	MED	HIGH	LOW	MED	HIGH	LOW	MED	HIGH	LOW	MED	HIGH
Stability at room temperature												
Baseline concentration : D0 (µg/mL)	1.0	10.8	30.7	0.9	11.3	31.8	1.0	11.0	31.8	1.0	10.0	31.7
48hrs (bias from D0, %)	1.0	-9.3	-4.8	-2.1	-8.6	-3.7	-6.9	-11.2	-5.9	-7.1	-7.1	-11.4
72hrs	1.0	-9.0	-8.9	-3.2	-4.4	-8.0	4.9	-11.2	-10.7	-11.1	-19.4	-20.4
1mth	3.1	-7.5	-2.7	3.2	-3.5	-8.2	-3.9	-6.2	-2.8	n/a	n/a	n/a
3mths	7.2	0.2	1.7	-27.7	-36.8	-26.9	6.8	-3.9	-2.4	n/a	n/a	n/a
Fridge stabiliy (+4°C)												
Baseline concentration : D0 (µg/mL)	1.1	10.2	31.3	1.0	10.5	32.0	1.1	10.4	32.4	1.0	9.9	31.6
48hrs (bias from D0, %)	-9.7	-2.3	-7.6	-7.6	-2.2	-5.4	-10.9	-3.8	-6.3	-6.9	-1.6	-4.7
1mth	2.8	3.2	-2.9	2.4	4.5	5.4	-3.2	2.5	0.6	1.9	-5.4	-7.2
3mths	7.3	1.5	-0.3	-18.3	-14.8	-16.1	5.5	-0.1	-7.4	-20.8	-19.0	-24.3
freezer stability (-20°C)												
Baseline concentration : D0 (µg/mL)	1.1	10.7	30.6	1.0	11.2	31.2	1.1	11.1	31.9	1.0	10.5	30.7
48hrs (bias from D0, %)	-7.4	-7.6	-4.5	-6.8	-6.7	-3.1	-7.5	-10.1	-7.7	-3.9	-7.5	-6.1
1mth	-1.9	-3.3	-1.4	-8.7	-3.9	-3.1	-1.9	1.0	1.3	6.9	-2.7	-3.6
3mths	4.5	-8.9	-5.2	-9.7	-11.8	-9.5	7.3	-8.8	-3.6	-1.0	-9.5	-3.4

n/a: not applied. n: number of replicates. D0: day 0 (baseline concentration).

Table 5. Pharmacokinetic parameters of LVX, CPX, MOX and RIF using WinNonLin® software

Antibiotic (number of patients)	**Rifampicin (n=12)**	**Levofloxacin (n=6)**	**Ciprofloxacin (n=6)**	**Moxifloxacin (n=3)**
Dose (PO)	600 mg BID	500 mg BID	750 mg BID	400 mg QD
Median Cmax [range value] (µg/mL)	11.7 [4.6-36.8]	9.1 [4.8-31.3]	2.7 [1.4-4.6]	4.9 [4.2-5.4]
Median Cmin [range value] (µg/mL)	0.5 [0.3-4.5]	3.2 [1.6-16.5]	0.6 [0.2-1.1]	1.2 [0.3-1.3]
Median Tmax [range value] (h)	2 [1-5]	2 [1-5]	2 [1-5]	2 [2-5]
Median AUC0-12 [range value] ($\mu g.h^{-1}.mL^{-1}$)	49.5 [17-180]	/	/	/
Median AUC0-24 [range value] ($\mu g.h^{-1}.mL^{-1}$)	/	152.5 [82-553]	35 [16-61]	36 [33-75]
Median AUC0-24/MCI [range value]	/	610 [168-2212]	341.5 [180-733]	144 [132-300]

AUC: area under the curve. MCI: minimum inhibitory concentration.