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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34

Abstract

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

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

46 RESULTS: Analysis time was 5 minutes per run, and all analytes and internal standards
47 eluted within 0.85-1.69 minutes. The calibration curves were linear over the range from 0.5 to
48 30 µg/mL for levofloxacin, ciprofloxacin, moxifloxacin and rifampicin with linear regression
49 coefficients above 0.995 for all analytes. The intra-day and inter-day coefficients of variation
50 were below 10% for lower and higher concentration. This method was successfully applied to
51 drug monitoring in patients with an osteoarticular infection.

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

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

57 **1. Introduction**

58 Osteoarticular infections (OAIs) are a fairly common type of infection with an incidence of
59 54.6 cases per 10,000 persons in France [1]. OAIs have variable expressions depending on the
60 context and age of patients. However, management follows common principles: identification
61 of the infectious agent, mapping of osteoarticular involvement and implementation of
62 prolonged antibiotic therapy [2]. The choice of antibiotic therapy is essentially based on the
63 ability of antibiotics to distribute to the infected site. The use of antibiotics with good bone
64 penetration therefore is a priority. Fluoroquinolones (FQs) such as levofloxacin (LVX),
65 ciprofloxacin (CPX), moxifloxacin (MOX) and rifampicin (RIF) member of the rifamycin
66 agents, show very good bone diffusion [3] and are frequently used in this context. Moreover,
67 given the high risk of resistant mutant selection when using these antibiotics as monotherapy,
68 and in order to ensure the widest possible coverage spectrum, both FQs and RIF should
69 always be used in combination in another antibiotic. The association between FQs and RIF is
70 very common in OAIs, especially during infection due to Gram positive bacteria, hence the
71 relevance of the ability to measure them simultaneously in patient plasma. In addition, given
72 their high intra- and inter-individual pharmacokinetic (PK) variability, the risk of drug
73 interaction and the need to limit the occurrence of resistance due to improper dosing,
74 therapeutic drug monitoring (TDM) of these antibiotics is highly recommended [4,5]. FQs
75 and RIF are concentration-dependent bactericidal antibiotics [6]; their TDM is primarily
76 based on the determination of their area under the curve (AUC). The AUC_{0-24} / minimum
77 inhibitory concentration (MIC) ratio for FQs and AUC_{0-12} for RIF are the major parameters
78 for predicting the clinical and microbiological efficacy of these antibiotics. The recommended
79 therapeutic efficacy threshold for FQs is defined in the literature as a AUC_{0-24} / MIC ratio >
80 125 for Gram negative bacteria, and > 35 for Gram positive bacteria [7–9]. The recommended
81 therapeutic range for RIF corresponds to an AUC_{0-12} 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.

107 Calibration range and quality control (QC) samples were prepared in blank human plasma by
108 adding the appropriate amount of working solutions of FQs mix and RIF. For LVX, CPX,
109 MOX and RIF, calibration range concentrations were 0.5, 1, 5, 10, 20 and 30 µg/mL,
110 respectively, and QC levels were 1, 10 and 30 µg/mL. A solution of mix of internal standard
111 (IS mix) at 100 ng/mL for LVX-d8, and at 500 ng/mL for CPX-d8, MOX-d5 and RIF-d8 was
112 prepared in methanol. All prepared solutions, calibration range and QC samples were stored at
113 -20°C. RIF solutions were stabilized by supplementation with an adequate volume of
114 ascorbic acid solution (pH 2.75; 0.11M) [20].

115 2.3. Instruments and analytical conditions

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

127 2.4. Samples pre-treatment

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

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

133 2.5. Validation

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

138 2.5.1. Selectivity

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

141
142 2.5.2. Carry-over

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

146 2.5.3. Linearity

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

151 2.5.4. Precision and accuracy

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

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

155 The inter-day assay was determined by repeating each QC sample twice a day over 15
156 different days. The concentration of each sample was determined using calibration standards
157 prepared on the same day. The precision was calculated as the coefficient of variation (CV,
158 %) within a single run (intra-day assay) and between different assays (inter-day assay) and the
159 accuracy as the bias between nominal and measured concentration. The acceptance limits
160 were CV < 15% for precision and within ± 15% of the nominal concentration for accuracy.

161 #### 2.5.5. Lower limit of quantification

162 To determine the LLOQ of LVX, CPX, MOX and RIF, serial dilutions in blank human
163 plasma, of the lowest point of the calibration range (0.5 µg/mL) were prepared to obtain 9
164 samples at the respective concentration of 0.45, 0.40, 0.35, 0.30, 0.25, 0.20, 0.15, 0.10 and
165 0.05 µg/mL. The precision and accuracy were evaluated using 10 replicates of each sample on
166 the same day (intra-day assay), and for 3 consecutive days (inter-day assay; n = 30). The
167 concentration of each sample was determined using calibration standards prepared on the
168 same day. The LLOQ corresponds to the lowest concentration sample with a CV < 20% and
169 within ± 20% of the nominal concentration.

170

171 #### 2.5.6. Matrix effect and extraction recovery

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

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

181 #### 2.5.7. Stability

182 Stability in plasma of LVX, CPX, MOX and RIF was tested by comparing the observed bias
183 between baseline concentration of three QC samples (1, 10 and 30 µg/mL) and the mean
184 concentration obtained after different storage conditions using freshly prepared calibrators.
185 The stability of the analytes in the plasma was tested immediately after sample preparation
186 (baseline) and after storing them at room temperature, +4°C and -20°C. Concentration of each
187 analyte was then determined after 24, 48 and 72 hours, 1 and 2 weeks and 1 and 3 months.
188 The stability after three freeze and thaw cycles was also tested. For this purpose, samples
189 stored for a minimum of 12 hours at -20°C, were kept at room temperature for at least 30
190 minutes followed by freezing in -20°C for a minimum of 12 hours.
191 All stability tests were done in triplicate per QC level. For each molecule, it was considered to
192 be stable in plasma sample when mean measured concentrations within ± 15% of the baseline
193 concentration.

194

195 #### 2.5.8. Dilution integrity

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

202 ### 2.6. Clinical application

203 Blood samples were obtained in patients with an osteoarticular infection hospitalized in the
204 orthopedic surgery department at Pitié-Salpêtrière who were treated, by oral administration,
205 with LVX or CPX or MOX and / or RIF. Blood samples were collected into heparin tubes
206 before dosing (t_0) and 1, 2 and 5 hours post-dosing at steady state. The median time of blood
207 collection was 5 (range: 3-12) days after start of treatment. AUC were performed using
208 WinNonLin® 4.1 software. AUC_{0-12} and AUC_{0-24} were estimated considering that, at steady
209 state, the 12 or 24 hours post-dosing concentration was equal to the before dosing
210 concentration (t_0). Plasma samples were prepared by centrifuging collected blood samples for
211 5 min at 4,500 g at room temperature. All plasma samples were frozen at -20°C until analysis.
212 French regulations on non-interventional observational studies do not require patient's
213 consent when analyzing data obtained from routine care. Approval for data collection was
214 obtained from the Commission Nationale de l'Informatique et des Libertés (n°1491960v0).
215

216 **3. Results**

217

218 3.1. Optimization of LC-MS/MS conditions

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

225 3.2. Method validation

226 3.2.1. Selectivity and carry-over

227 Six plasma samples without LVX, CPX, MOX and RIF but containing the following drugs:
228 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

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

254 ### 3.2.5. Matrix effect and extraction recovery

255 Matrix effect and extraction recovery for all the analytes ranged from 93.4% to 108.3% and
256 91.2% to 105.7%, respectively, and were constant over the concentration range for each of
257 them, as shown in Table 3. The result of ME indicated that there was no significant ionization
258 suppression or maximization resulting from sample matrices. Moreover, the method resulted
259 in high recovery value at all QCs showing good efficiency.

260 ### 3.2.6. Stability

261 Table 4 shows the stability in plasma. The data presented correspond to the bias between the
262 mean measured concentration and the baseline concentration of LVX, CPX, MOX and RIF
263 using different test conditions. LVX and MOX remained stable for 3 months regardless of the
264 storage temperature. CPX remained stable for 3 months at -20°C; 1 month at room
265 temperature and +4°C. Stabilized RIF (with ascorbic acid) remained stable for 3 months at -
266 20°C; 1 month at +4°C and only 48 hours at room temperature.
267 Regarding freeze and thaw stability, QC samples were stable after three freeze and thaw
268 cycles. All samples were measured with inaccuracy (bias from baseline concentration, %)
269 ranging from -1.3% to -6.1% for all analytes at all tested concentrations and with imprecision
270 (CV, %) ranging from 2.1% to 4.5% for all analytes at all tested concentrations.

271 ### 3.2.7. Dilution integrity

272 The inaccuracy (bias, %) and imprecision (CV, %) of the diluted samples were: LVX (-1.4;
273 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

298 quantification of 20 anti-tuberculosis drugs including LVX, CPX, MOX and RIF [17] with a
299 calibration range from 0.2 to 10 µg/mL for these four antibiotics. Likewise, Jourdil et al. have
300 also developed a method for quantification of 9 antifungals and antibiotics drugs including
301 LVX, CPX and RIF [18] with a calibration range from 0.04 to 6 µg/mL for LVX and CPX,
302 and from 0.2 to 8 µg/mL for RIF, respectively. These two methods might be not suitable for
303 the measurements of the C_{max} reported for RIF and LVX in clinical practice. In addition, in
304 our method, in case of concentrations exceeding our ULOQ, especially for LVX and RIF, the
305 dilution integrity test shows that the sample can be diluted in blank plasma without affecting
306 analysis quality.
307 Concerning the sample preparation and run time analysis, in the method developed by Kim et
308 al. [17], the sample pretreatment was similar to ours (protein precipitation with methanol
309 containing ISs) but the run time was 9 min. Our method is faster (run time of 5 min) which
310 could reduce the time required for quantification of large number of samples. In the method
311 developed by Jourdil et al. [18], the run time was 4 min but, MOX was not quantified.
312 Furthermore, with respect to other methods, our method is the only one in which LVX, CPX,
313 MOX and RIF are analyzed with respect to their respective isotopically internal standard.
314 Regarding stability, we performed a 3-month stability study. Lee et al. showed in their study
315 that LVX and MOX were stable for 3 months at -80°C in human serum [19]. We have shown
316 in our study that this was also the case at -20°C in human plasma. Furthermore, to our
317 knowledge, our study is the first to demonstrate the stability of RIF stabilized by ascorbic acid
318 for 3 months at -20°C in human plasma. In their study, Kim et al. [17] showed a degradation
319 of RIF (studied without the addition of ascorbic acid) in plasma after storage at -80°C for 3
320 months. Our study shows that the addition of ascorbic acid greatly improves the stability of
321 RIF in plasma. These stability data are important information for the storage of patient plasma

322 samples. This is particularly important in the case of clinical research protocols, where
323 samples may need to be stored for a long time before they can be assayed.
324 Concerning the clinical application, 3 of 12 patients taking RIF had a C_{max} and an AUC_{0-12}
325 below the expected minimum values of 8 µg/mL and 30 $µg.h^{-1}.mL^{-1}$, respectively, and 4 of 12
326 patients had an AUC_{0-12} above the expected maximum values of 65 $µg.h^{-1}.mL^{-1}$. Moreover, as
327 shown in Table 5 and Figure 2, there is a very large inter-individual pharmacokinetic
328 variability of FQs and RIF in patients treated for osteoarticular infection. In this context,
329 TDM could be a powerful technique to measure and adjust the dose, which is especially
330 important for concentration-dependent bactericid antibiotics such as FQs and RIF. Using this
331 method, 8 out of 27 patients were actually able to benefit from a dose adjustment based on
332 their pharmacokinetic profiles. In the end, the method developed could be used in a PK/PD
333 study to support the relevance of TDM in osteoarticular infections.
334

335 **5. Conclusion**

336 We have developed and validated a rapid, selective, simple, accurate, precise and reliable LC-
337 MS/MS method for the simultaneous quantification of levofloxacin, ciprofloxacin,
338 moxifloxacin and rifampicin in human plasma. This technique is currently used in clinical
339 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

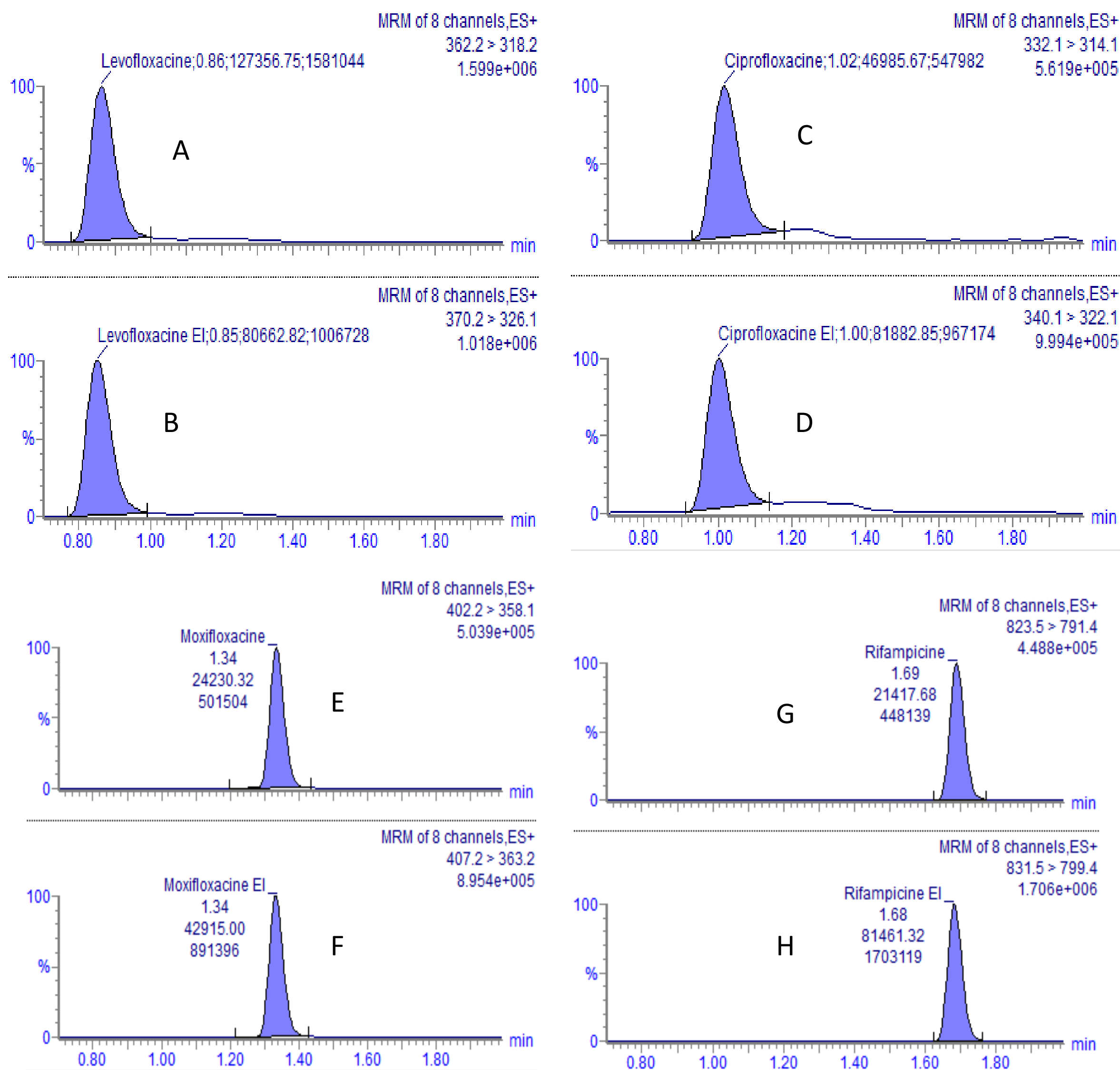


Figure 1.B blanks plasma of (A) LVX, (B) LVX-d8, (C) CPX, (D) CPX-d8, (E) MOX, (F) MOX-d5, (G) RIF, (H) RIF-d8

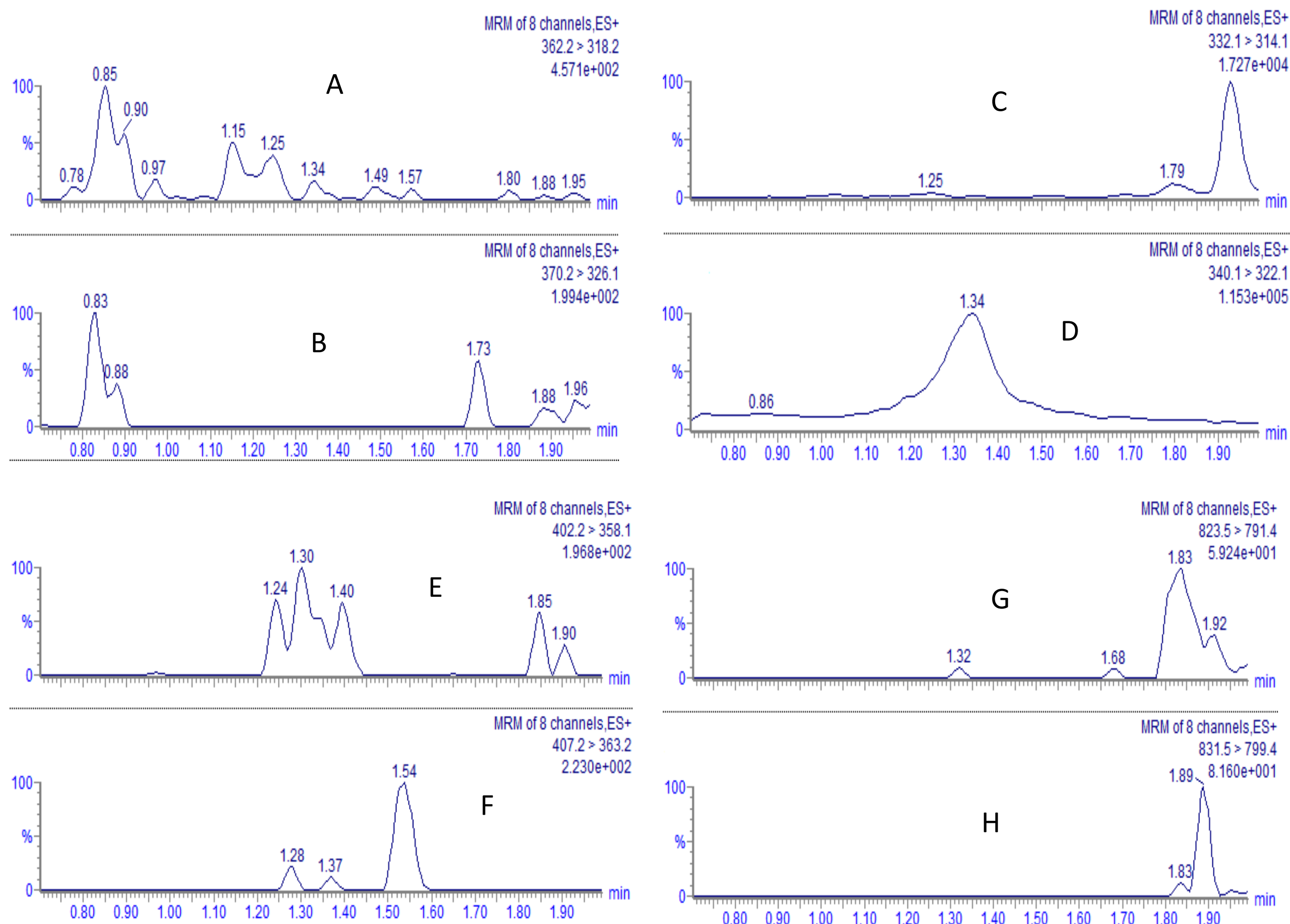
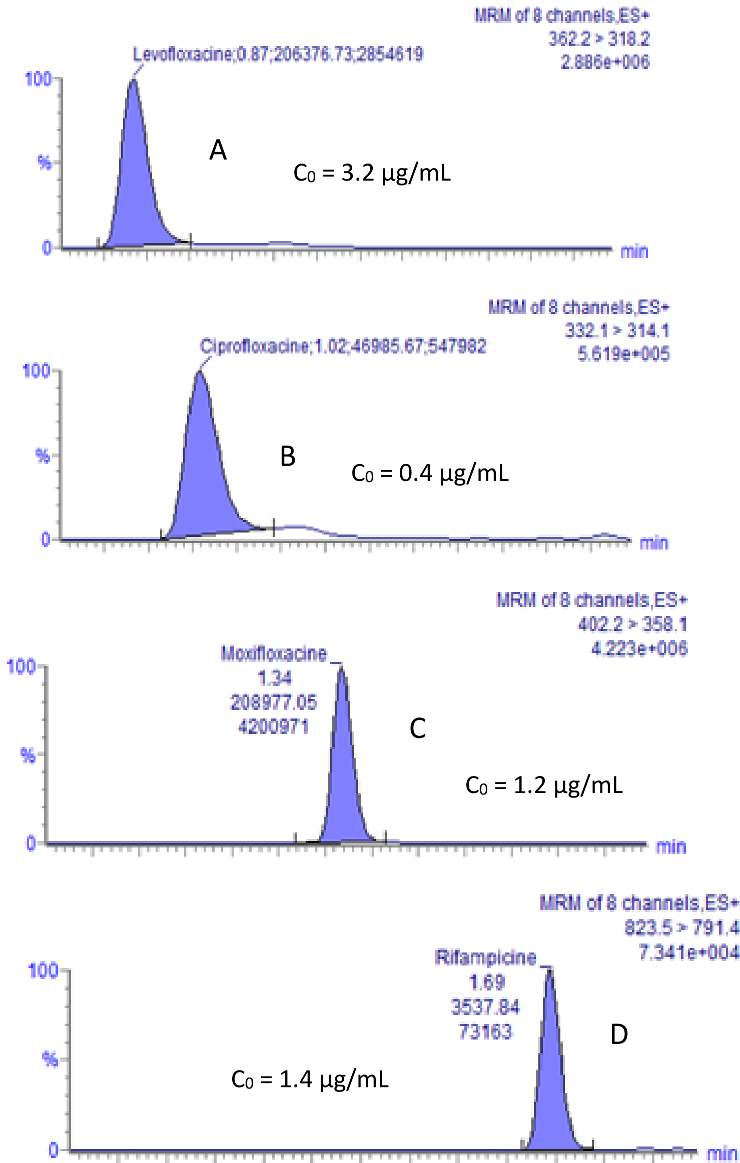
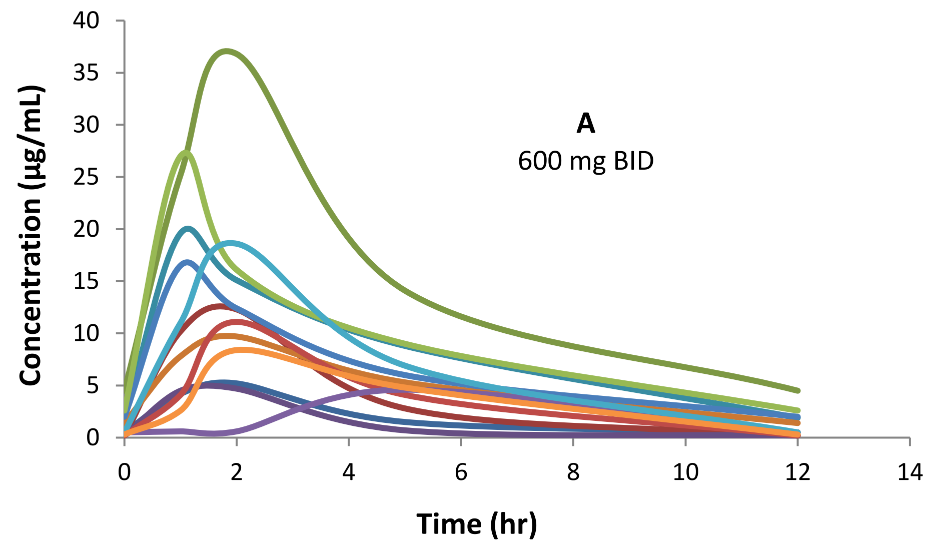
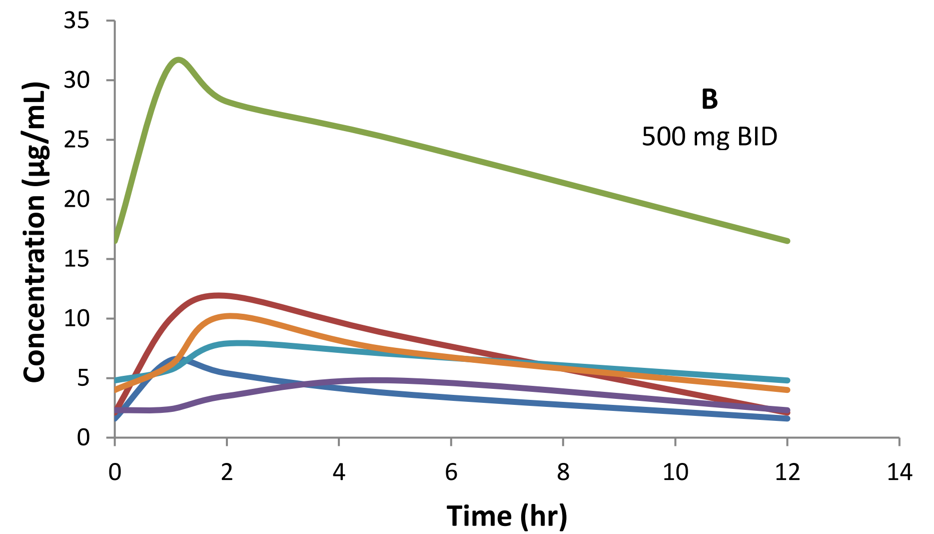


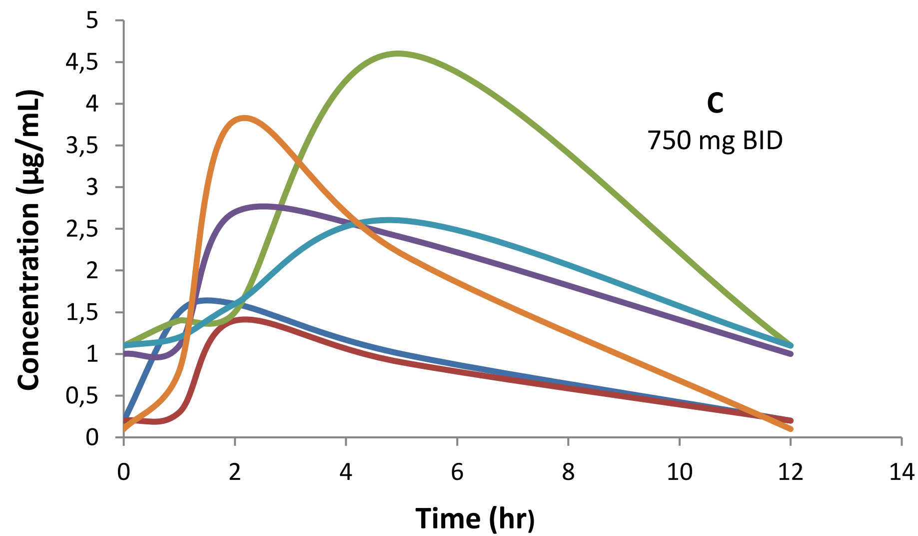
Figure 1.C Chromatograms of patients sample at t_0 of (A) LVX, (B) CPX, (C) MOX, (D) RIF.



C_0 : Concentration of patient sample at t_o







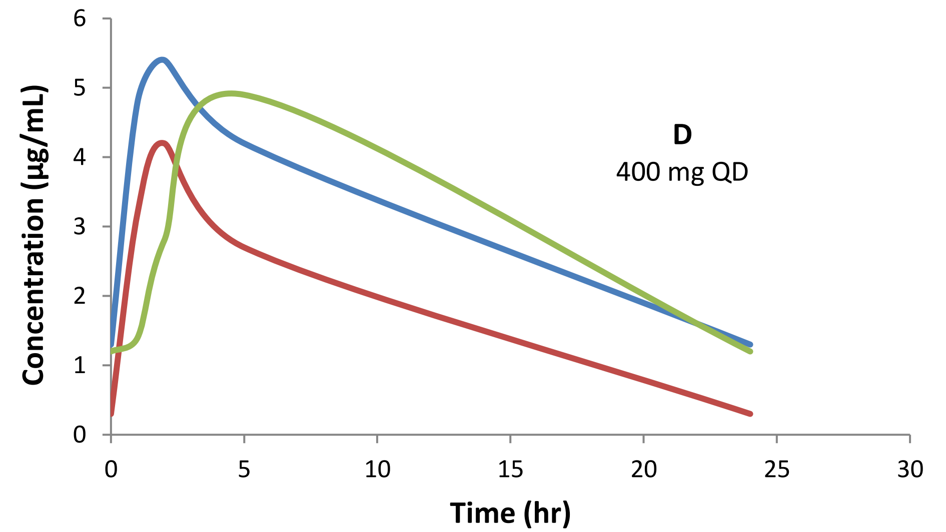


Figure 2. Concentration versus time profile of (A) rifampicin (N=12), (B) levofloxacin (N=6), (C) ciprofloxacin (N=6) and (D) moxifloxacin (N=3)

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 (%)
LVX	1	0,96	0,01	0,8	-3,6	0,99	0,03	2,9	-1,5
	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
CPX	1	0,98	0,01	1,1	-2,0	0,97	0,04	4,6	-3,2
	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
MOX	1	0,96	0,01	1,1	-4,1	1,05	0,06	5,4	5,3
	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
RIF	1	0,90	0,01	1,0	-10,0	0,99	0,03	3,3	-0,7
	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.