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Antibiotic and efflux: Combined spectrofluorimetry and mass spectrometry to evaluate the involvement of concentration and efflux activity in antibiotic intracellular accumulation

Estelle DUMONT[1,#], Julia VERGALLI[1,#], Laurence CONRAUX[2,#], Carine TAILLIER[2], Aurélie VASSORT[2], Jelena PAJOVIĆ[3], Matthieu RÉFRÉGIERS[3], Michael MOUREZ[2], Jean-Marie PAGÈS[1,*]

[1]UMR_MD1, Aix-Marseille Univ, U-1261 INSERM, SSA, IRBA, MCT, Marseille, France

[2]Infectious Diseases Therapeutic Area, Sanofi R&D, Marcy l'Etoile, France

[3] DISCO beamline, Synchrotron Soleil, Saint-Aubin, France

[#]These authors contributed equally to this work

[*]Corresponding author:

J.-M. Pagès, UMR_MD1, U-1261 INSERM, Membrane et Cibles Thérapeutiques, Faculté de Médecine et Faculté de Pharmacie, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France.

Tel:(33) 491324587. Fax:(33) 491324606. E-mail: jean-marie.pages@univ-amu.fr

Running title: Combined methods for studying antibiotic transport

Synopsis

Background: In Gram-negative bacteria, passing through the double membrane barrier to reach the inhibitory concentration inside the bacterium is a pivotal step for the antibiotic activity. Spectrofluorimetry has been developed to follow the fluoroquinolone accumulation inside bacteria using intrinsic bacterial fluorescence as an internal standard. However, adaptation for non-fluorescent antibiotics is needed; quantitative methods based on mass spectrometry offer the possibility of expanding the detection range obtained by spectrofluorimetry.

Objectives: We wanted to validate with fluorimetry the use of mass spectrometry to measure antibiotic accumulation in cells and to determine the relationship between antibiotic concentrations and the amount of intra-bacterial accumulation in different efflux backgrounds on the same batch of molecules.

Methods: Spectrofluorimetry was performed in parallel with mass spectrometry on the same samples to measure the ciprofloxacin and fleroxacin accumulation in cells expressing various efflux pump levels. A microplate protocol was set up to determine the antibiotic accumulation as a function of external antibiotic concentrations.

Results: A consistency existed between the data obtained with spectrofluorimetry and mass spectrometry, whatever the activity of efflux pump or the tested antibiotic. The results highlighted a different dynamic of uptake between ciprofloxacin and fleroxacin as well as the relationship between the level of efflux activity and antibiotic accumulation.

Conclusions: We have developed a microplate protocol and cross-validated two complementary methods, spectrofluorimetry that contains a solid internal standard and mass spectrometry that allows detection of low antibiotic amounts. These assays allow studying the dose-effect and the efflux impact on the intra-bacterial accumulation of antibiotics.

Introduction

A main challenge in bacterial chemotherapy is to determine and carefully use the *in situ* parameters that modulate the activity of drugs in order to improve antibacterial efficacy.[1,2] This is particularly important with the continuing emergence and the spread of MDR bacteria that contribute to therapeutic failure.[3–6] Several papers illustrate the intensity and relevance of the concern and it is absolutely required to understand membrane permeation and intracellular concentration of antibiotics in clinical isolates: crossing the double membrane barrier to reach a threshold concentration is a pivotal step of antibacterial action.[7–15] This key point has not been strongly addressed until now or only in few specific cases with characterized bacterial strains.[10,13,16,17]

As recently reported[10,18,19], there is a large collection of methods available to measure efflux activity, but their sensitivity and validity must be clearly demonstrated under different conditions. Thus, the lack of appropriate assays containing well-defined internal controls that allow standardization of signals obtained with various strains under various conditions is a serious bottleneck for medicinal chemistry efforts aiming for the optimization of an antibiotic intracellular accumulation. Mass spectrometry appears as a promising technique to determine the intracellular accumulation of label-free compounds. [20–24] Ultimately, this approach might help to characterize the membrane permeation of any potential drugs, quantify antibiotic accumulation kinetics with appropriate internal controls and meet the throughput requirements of optimization programs based on structure-activity relationship. In the case of fluorescent drugs, intracellular accumulation can be monitored using a synchrotron light source and use the intrinsic fluorescence of bacterial amino acids as a powerful internal standard to correct for biological variations associated with bacterial samples.[25] For example, the fluoroquinolone antibiotic, fleroxacin, exhibits sufficient changes in fluorescence intensity to monitor its

accumulation in single bacterial cells.[25,26] Recent results allowed us to validate this method with a multidrug resistant clinical strain of *Enterobacter aerogenes* and an efflux pump deficient (Δ*tolC*) derivative of this strain, demonstrating the importance to use a robust internal control.[27]

The Innovative Medicine Initiative-funded consortium Translocation[28] (www.imi.europa.eu/content/translocation) has defined as its main objective the study of antibiotic translocation across bacterial membranes.[17] The consortium uses, new approaches, and more traditional ones, to improve our understanding of drug transport and to support future strategies in order to face the antibiotic resistance crisis. In this context, it is important to define common standards and/or protocols to validate the new methods and for future correlation with the antibacterial activities.[10,29] This is the main objective of this study that proposes a joint protocol for spectrofluorimetry and mass spectrometry analyses associated to biological/biochemical characterizations of bacterial samples. From this work, a new perspective emerges on how to measure kinetics of antibiotic uptake inside bacterial cells and correlate them to the antibiotic mode of action in order to (i) understand and combat the emergence and spread of resistant strains and (ii) extend the activity of antibiotics or (iii) upgrade the design of new molecules. In addition, this study contributes to the recently proposed concepts, "Resident Time Concentration Close to Target" (RTC2T) and "Structure Intracellular Concentration Activity Relationship" (SICAR).[10]

Materials and methods

Bacteria and Media

Escherichia coli strains used in this study are listed in **Figure 1**. These isogenic strains have been previously described regarding their antibiotic susceptibility.[30]

Immunodetection

Exponential-phase bacteria in liquid Mueller Hinton II medium were centrifuged and the pellet was solubilized in loading buffer.[31] Total cell protein (OD_{600} = 0.02 corresponding to equal protein per well) was loaded onto a SDS-polyacrylamide gel (10% polyacrylamide (w/v), 0.1% SDS (w/v)). Proteins were electro-transferred onto nitrocellulose membranes in transfer buffer. An initial saturating step was performed overnight at 4°C with Tris-buffered sodium (TBS: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing skimmed milk powder (10%). The nitrocellulose sheets were then incubated in TBS + skimmed milk powder + Triton X-100 (0.2%) for 2 h at room temperature in the presence of polyclonal antibodies (1:5,000, 1:2,000 and 1:10,000 dilution for anti-AcrA, anti-AcrB, OmpC and OmpF respectively) directed against denatured AcrA, AcrB, OmpC and OmpF respectively.[31] The detection of antigen-antibody complexes was performed with horseradish peroxidase secondary antibody conjugated Immune-Star goat anti-rabbit IgG antibodies (BioRad) and revelation was performed using a ChemidocXRS+ (BioRad).

Drug susceptibility assays

Ciprofloxacin and fleroxacin were assayed to study the antibiotic susceptibility of *E. coli* strains. MIC values of antibiotics were determined by the microdilution method (CLSI) in liquid Mueller Hinton II medium by using the twofold standard microbroth dilution method

(microplates and automatic analyses Tecan®) (CLSI, http://clsi.org/). MIC values were read after 18 h of incubation at 37°C. Experiments were carried out in triplicate and the resulting medians were presented.

Accumulation protocol

Bacteria grown at 37°C in Luria-Bertani broth in its exponential-phase (0.6 optical density units at 600 nm) were concentrated 10-fold. The bacterial suspension was centrifuged at 6000 × g for 15 min at 20 °C and pellets were re-suspended in 1/10 of the initial volume in a sodium phosphate buffer (50 mM) at pH 7 supplemented with $MgCl_2$ (5 mM) (NaPi-$MgCl_2$ buffer) to obtain a density of 6.10^9 cfu.mL^{-1} (colony-forming-unit). In 96-well deep well plates, 1.44 mL of the bacterial suspension was incubated 2 or 10 min at 37°C (final volume 1.8 mL per well) with different concentrations of fleroxacin and ciprofloxacin (0 to 5 mg/L), in the absence or in the presence of the efflux blocker cyanide-m-chlorophenylhydrazone (CCCP) at 10 μM for the efflux pump overproducer strain AG102. CCCP collapses the energy-driven force needed by the efflux pump to expel antibiotics in the resistant strain.[32] Bacterial suspensions incubated without antibiotics, with or without CCCP, were used as controls. Suspensions (800 μL) were then loaded on 1 M sucrose cushions (1.1 mL) in two deep well plates (one for the spectrofluorimetry analysis and the other for the mass spectrometry analysis) and centrifuged at 2 700 × g for 20 min at 4°C to eliminate extracellular-adsorbed compounds and collect washed bacteria.[29] The same incubation mixtures were used to performed spectrofluorimetry and mass spectrometry analyses, in three replicates each. This accumulation assay was repeated three times.

To control that the bacterial cells are alive during the experimental time, the number of cfu was determined by sampling the bacterial suspension at 2 and 10 min during antibiotic incubation. The cfu numbers were determined and no change in cell viability was observed

during this period that corresponds to accumulation assay (data not shown). It must be noted that the ratio "bacterial cell/antibiotic concentration" was different in the MIC assay and in accumulation assay (about 10-20 times higher in accumulation conditions compared to MIC assays). Furthermore, the accumulation assay was carried out using starving conditions during a limited incubation time (2-10 min) as previously determined.[27]

Spectrofluorimetry assay

To follow the compound uptake by bacterial population, the fluorimetric assay previously described was used.[25] Pellets corresponding to 800 μL of bacterial suspensions were lysed with 500 μL of 0.1 M Glycin-HCl pH 3 buffer overnight at room temperature. The lysis efficacy has been checked by cfu determination as previously mentioned.[29] After a centrifugation in deep wells for 20 min at 2 700 × g at 4°C, 100 μL of lysates were analyzed by spectrofluorimetry at λ_{ex} 290 nm and λ_{em} around 450 nm for fleroxacin and λ_{ex} 275 nm and λ_{em} around 450 nm for ciprofloxacin. To quantify the fluoroquinolone fluorescence intensity in bacteria lysate, spectra were normalized using the tryptophan peak at 356 nm and subtracted from control sample spectra.[27] Fluoroquinolone concentrations in bacterial lysate were calculated according to a calibration curve (See paragraph 'Standard curves').[29]

Mass spectrometry determination

Sample preparation for Mass spectrometry analysis

Pelleted bacteria were thawed during 10 min at 37°C and resuspended in 200 µL of 0,1 M Glycin-HCl pH 3 buffer. Samples were sonicated during 5 min (sonic batch) and incubated at 37°C during 2 hours under gentle agitation. The lysis efficacy has been checked by cfu determination (data not shown). After a first centrifugation at 6,100 g (10 min, room temperature) 150 µL of the supernatant were recover and centrifuged a second time in the

same conditions. 40 µL of these final supernatants were then put in a 96 well plate for MS analysis.

Mass spectrometry analysis

Quantitative analysis was performed on a Waters ACQUITY UHPLC HSS T3 column (1.8 μ m 50 x 2.1 mm) at room temperature with the flow rate of mobile phase set at 0.5 mL/min. The mobile phase consisted of (A) 0.1 % formic acid in water, and (B) 0.1 % formic acid in 95 % acetonitrile, 5 % water. A linear gradient elution program was applied as follows: 0-1 min: 0-100 % B; 1-1.3 min: 100 % B; 1.3-1.4 min: 100 %-0 % B; 1.4-2 min: 0% B. The sample injection volume was 5 μ L.

All the mass spectrometry experiments were performed using a TSQ Quantiva mass spectrometer (Thermo Scientific, San Jose, CA, USA) interfaced with an UltiMate 3000 XRS UHPLC system (Thermo Scientific San Jose, CA, USA). Data was processed using Trace Finder version 3.3 (Thermo Scientific, San Jose, CA, USA).

Mass spectrometry detection was performed in positive ion mode and each drug was quantified using one selected reaction monitoring transition (dwell time ~ 50ms): m/z 332.141 -> 287.986 for the ciprofloxacin and 370.181 -> 326.111 for the fleroxacin. Details regarding collision energy and RF-lens values are presented in **Table 1**.

Standard curves

Mass spectrometry

Solutions of each compound for calibration were freshly prepared in bacteria lysate. From a first concentration of 5 mg/L, 4-time serial dilutions were done in bacteria lysate to a final concentration of 0.076 ng/mL, leading to 9 solutions used for mass spectrometry calibration curves (**Figure S1**).

Spectrofluorimetry

Various known concentrations of fleroxacin and ciprofloxacin were mixed with bacteria lysates at OD=4.8 and samples were measured with spectrofluorimeter (n=3) at λ_{ex} 290 or 275 nm respectively and λ_{em} around 450 nm. The trend lines and the slopes of the fleroxacin and ciprofloxacin standard curves were determined in the various studied bacteria lysates (**Figure S2**). The corrected fluorescence value of each spectrofluorimetry sample (corrected with the fluorescence signal from bacteria as previously described)[27] was divided by the slope of the trend line to obtain the fleroxacin and ciprofloxacin concentrations.

Results and Discussion

Fluoroquinolone susceptibility and AcrAB expression

The activity of fleroxacin and ciprofloxacin were assayed on *E. coli* AG100, AG100A, a derivative strain devoid of the AcrAB efflux pump, and AG102, a strain that overproduces AcrAB (**Figure 1**a). The same strains were used for both detection methods. In general, AG100A was more susceptible to the drugs than the wild type, while AG102 was more resistant. Immunoblots confirmed the absence of AcrAB in AG100A and a more intense signal in AG102 (**Figure 1**b). The porins, OmpC and OmpF, are present at an equivalent level of expression in the 3 strains (**Figure 1**b).

Mass Spectrometry versus spectrofluorimetry to evaluate the fluoroquinolone accumulation

In order to compare the two approaches for determining the antibiotic accumulation in various efflux backgrounds, an experimental protocol was designed (see **Figure S3)**. In this protocol, we generated duplicate samples for each experimental point (*e.g.* strain, time, drug concentration). Each sample was analyzed in triplicate (corresponding to 3 independent wells) using spectrofluorimetry or mass spectrometry method (blind assay).

Ciprofloxacin accumulation

The analysis was performed on measurements of ciprofloxacin concentrations accumulated in all studied strains (AG100, AG100A, AG102, AG102 incubated with CCCP) at 2 min and 10 min incubations (**Figure 2**). The consistency between spectrofluorimetry and mass spectrometry was quantified by Bland-Altman calculations (**Figure 2**). The difference is centered around zero with neither absolute systematic error nor strong dependency on the magnitude of measurements. The observed bias is relatively low (297 ± 5477 molecule/cell)

and not significant, and biases are similar between incubations at 2 min and 10 min (data not shown). Under the conditions used, the two methods give equivalent results.

Fleroxacin accumulation

The results are presented in **Figure S4**. As observed with ciprofloxacin, the Bland-Altman plot demonstrated the consistency of the measurements obtained with spectrofluorimetry and mass spectrometry (Bias of 263 ± 4008 molecule/cell). The difference is centered around zero and no proportional error is present in the comparison between the two methods.

It must be noted that only in the case of fluorimetry assay, as reported previously, the internal signal corresponding to intrinsic bacterial fluorescence (tryptophan fluorescence) was systematically used to correct the data about the variation of cellular masses presented in the various samples.[27,29] The fact that spectrofluorimetry measurements are corrected with the bacterial internal standard, unlike mass spectrometry data, can explain the ranges of agreement obtained on the Bland-Altman plots. Another specific point concerns the detection limit of spectrofluorimetry: it cannot be used to detect accumulations performed with concentration lower than 500 ng/mL under the conditions used. In contrast, mass spectrometry gives access to a wider range of low concentrations (down to 25 ng/mL) in our conditions.

Concentration effect, influx and efflux activity

The measure of the accumulation of compounds inside the *E. coli* AG100A strain devoid of AcrAB pump can be used to determine the respective efficacy of ciprofloxacin and fleroxacin uptake. The results presented in **Figure 3** indicate that ciprofloxacin is about 2-fold more efficiently accumulated compared to fleroxacin in the absence of AcrAB efflux. A quite similar value has been calculated using spectrofluorimetry assay and previously proposed for the concept SICAR^{IN}.[32] In this study, the mass spectrometry assay also shows a difference in uptake for ciprofloxacin and fleroxacin (Kruskal-Wallis test, $p<0.01$, $n=27$).

To evaluate the impacts of external concentration effect and/or of efflux level on the rate of intracellular accumulation, a large range of ciprofloxacin concentrations was incubated with the parental strain, the AcrAB- derivative and the AcrAB overproducer isogenic strains. The data obtained with mass spectrometry analyses are presented in **Figure 4.** A strong difference was observed between the accumulations in the different AcrAB strains: the maximal accumulation, whatever the concentration used, was obtained with AG100A or with AG102 in the presence of CCCP; in contrast the minima was obtained with AG102 ($p<0.001$). Moreover, the overproduction of AcrAB in AG102 has a noticeable effect on ciprofloxacin accumulation at high concentrations (3.75 and 5 µg/mL) compared to the pump basal expression obtained in AG100 ($p<0.05$).

The fold-increase (FI) was defined as the ratio: F*[AG102+CCCP]* / F*[AG102]*, where F*[AG102+CCCP]* corresponds to the ciprofloxacin accumulated in AG102 in the presence of CCCP and F*[AG102]* corresponds to the ciprofloxacin accumulated in the same strain in the absence of CCCP. The F*[AG100A]* / F*[AG102]* was also calculated and the different ratios were plotted for the given external ciprofloxacin concentration in **Figure 5**. Interestingly, with increasing ciprofloxacin concentration, a significant decrease ($p<0.05$) of FI [*AG102+CCCP /AG102*] and FI [*AG100A/AG102*] was observed (from about 4.4 to 2.7 and 3.6 to 2.4, respectively)(**Figure 5**). These results suggest that a saturation of the efflux ability to expel the internalized molecules can be reached in the *E. coli* strain that overproduced AcrAB when high concentration of ciprofloxacin is used.

These data explain the key contribution of efflux pump in fluoroquinolone resistance in clinical strains where the uptake of antibiotic can be impaired by alteration of the membrane permeability as previously hypothesized.[10]

Conclusion

A variety of methods has been previously reported to follow the intracellular accumulation of antibiotic in enterobacterial cells that feature membrane-associated mechanisms of resistance affecting many or all antibiotic classes, including reduction of outer membrane permeability or increase of levels of efflux pumps .[10,18] Until now and despite recent methodology efforts, no comparative assays have been carried out in order to have an independent validation of the proposed protocols or at least to check their consistency and robustness.

In this study, our aim was to determine the internal concentration of fluoroquinolones, ciprofloxacin and fleroxacin and to measure the effect of antibiotic concentrations on bacteria expressing various levels of AcrB pump, using fluorimetric and mass spectrometric (LC&MS/MS) methods.

For the first time, a robust consistency was described between these two approaches, spectrofluorimetry and mass spectrometry, whatever the efflux activity, the molecule or the concentration of antibiotic used in the assays. Moreover, by combining the spectrofluorimetry measurement, that contains a solid internal standard[29], with LC&MS/MS determination, that allows studying low concentrations of molecule, these two methods can be viewed as complementary. Our study is especially important in view of recent papers reporting the accumulation of antibacterial molecules using mass spectrometry assay when this assay lacks robust internal control for normalization.[23]

The development of LC&MS/MS assays in microplates allows to determine the relationship between a large range of antibiotic concentrations and the amount of intra-bacterial accumulation in different efflux backgrounds without and with the presence of an efflux poison (CCCP). Importantly, the data demonstrate that ciprofloxacin penetrates and

accumulates more than fleroxacin in an AcrAB- strain The measurement of uptake in the absence of efflux activity, defined by $SICAR^{IN}$ as previously proposed,[32] is thus confirmed. With increasing external concentrations of compound, the effect of efflux on internal accumulation was altered and the accumulation ratio obtained between efflux- and efflux ++ strains was significantly decreased, showing that efflux can be saturated. This effect must be taken into consideration when assessing the impact of efflux on new antibacterial agents.

In addition, the fact that efflux pumps can indeed be saturated supports the strategy previously proposed by several authors to generate a substrate competition on the binding sites of efflux pumps by using molecular lures or adjuvants to restore the intracellular antibiotic concentration.[31,33–36]

Intrinsic bacterial fluorescence has been previously used to standardize/normalize the data in the quantification assays for fluorescent drugs.[25] In this study, the good correlation between mass spectrometric determination and fluorimetric analyses demonstrated the advantage of this internal measure for monitoring the efficacy of lysis protocol and correcting the crude signals for protocol validation.[10,19]

In the future, it will be necessary to develop additional assays using similar methods as the one described here to measure in whole bacteria the apparent affinities of various antibiotics for the different bacterial transporters involved in the influx step (*e.g.* porins) or involved in the efflux (*e.g.* other AcrAB family members).

To conclude, with this method the concept of "structure intracellular concentration activity relationship" (SICAR) previously proposed[10,19] is now applied to a large concentration range. In SAR studies for biologically active molecules, SICAR will correlate the chemical structure to the efficacy of translocation through bacterial membrane and the resulting intracellular

accumulation; while taking into account recently proposed key parameters, such as globularity and charge, as reported by Richter *et al.*[23] Thus, the role of chemical side chains involved in the membrane permeation (IN) or in the efflux transport (OUT) can be dissected and allow the design of drug that fit better the translocation constraints present in resistant clinical isolates.

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Transparency declarations

The authors (L.C., C.T., A.V. and M.M.) declare competing financial interests: L.C., C.T., A.V. and M.M. are former/current employees of Sanofi R&D. All other authors: none to declare.

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Figures

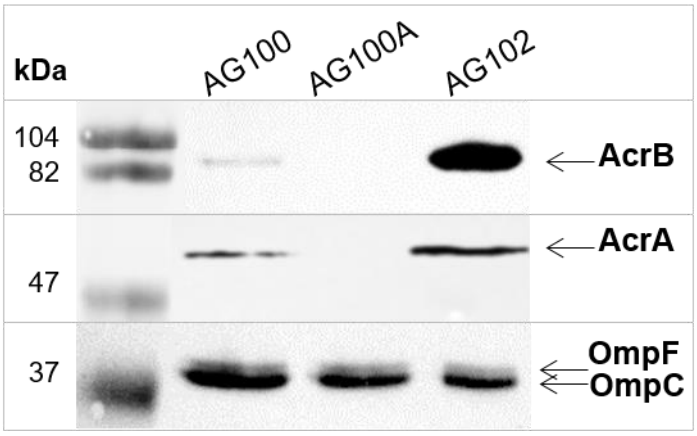
Figure 1. Expression of AcrAB efflux components and porins, and fluoroquinolone susceptibilities of the studied bacterial strains.

a

E. coli strains	Detected porins		Efflux pump	MIC[a]	
	OmpC	OmpF	(AcrAB)	Fleroxacin (mg/L)	Ciprofloxacin (mg/L)
AG100*	+	+	AcrAB+	0.125	0.016
AG100A*	+	+	AcrAB-	0.03-0.06	0.004-0.008
AG102*	+	+	AcrAB++	0.5	0.062

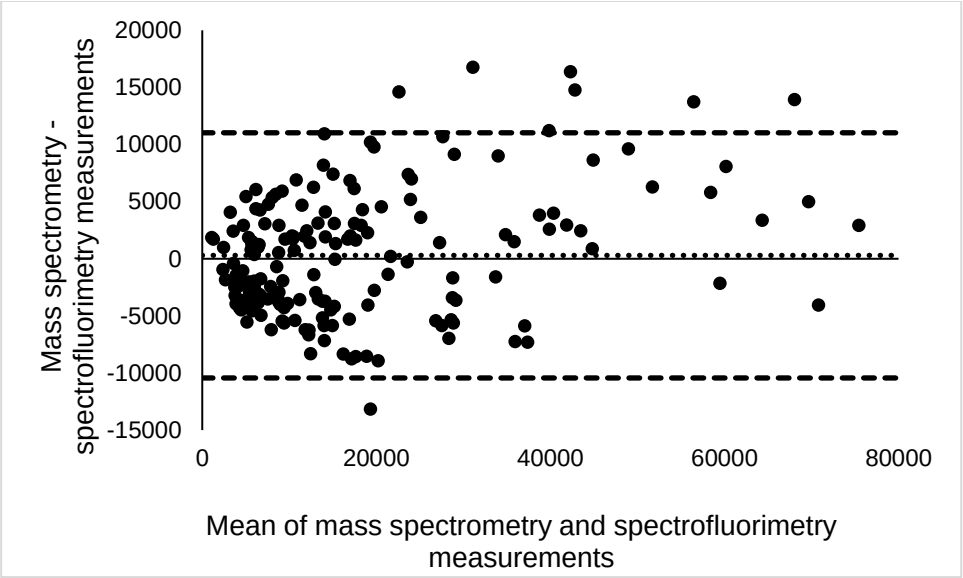
[a] Strains and susceptibilities have been previously reported.[30]

b



(**a**) Fluoroquinolone susceptibilities of AG100, AG100A and AG102. (**b**) Western blot showing AcrB, AcrA and porins OmpC and OmpF in AG100, AG100A and AG102 grown in MH2.

Figure 2. Bland and Altman plot for mass spectrometry and spectrofluorimetry measurements of ciprofloxacin accumulation.



Ciprofloxacin was accumulated for 2 min and 10 min in AG100 (AcrAB+), AG100A (AcrAB-), AG102 (AcrAB++) and AG102 cells incubated in the presence of CCCP (10 µM). The ciprofloxacin intracellular concentrations (molecules/cell) obtained in the various isogenic strains were determined with the spectrofluorimetry and mass spectrometry methods. The figure shows the Bland-Altman plot of the differences between mass spectrometry and spectrofluorimetry quantification against the averages of the two methods with bias of 297 ± 5477 units. The bias is represented by the gap between the X axis, corresponding to a zero difference, and the parallel dotted line to the X axis. The 95% limits of agreement corresponding to +/- 1.96 SD are represented in dashed lines.

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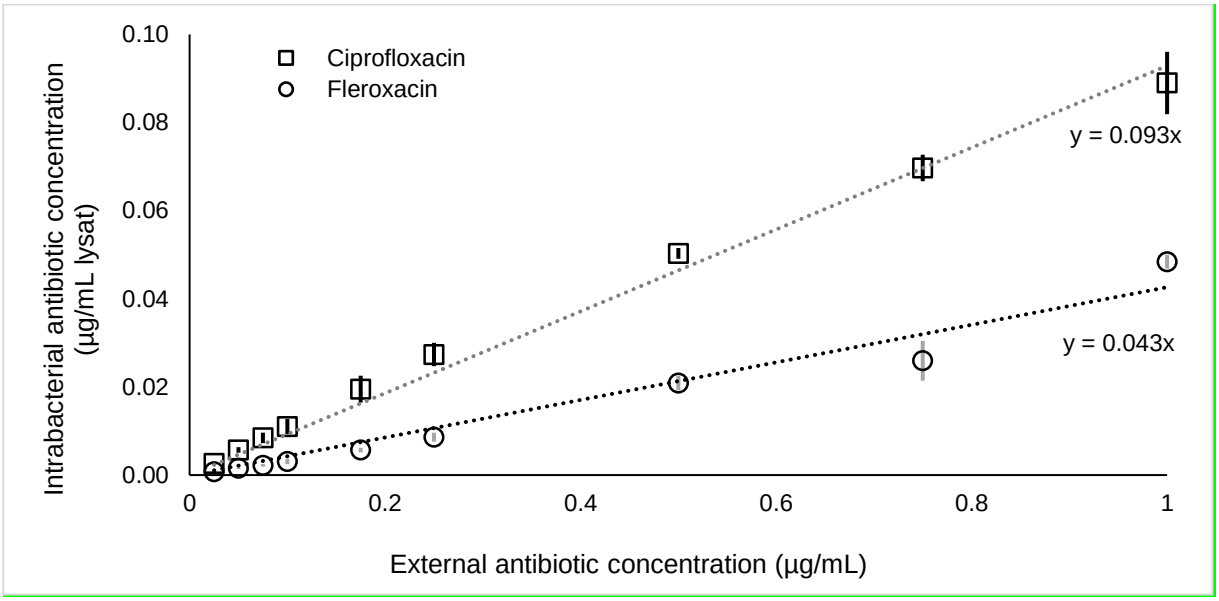
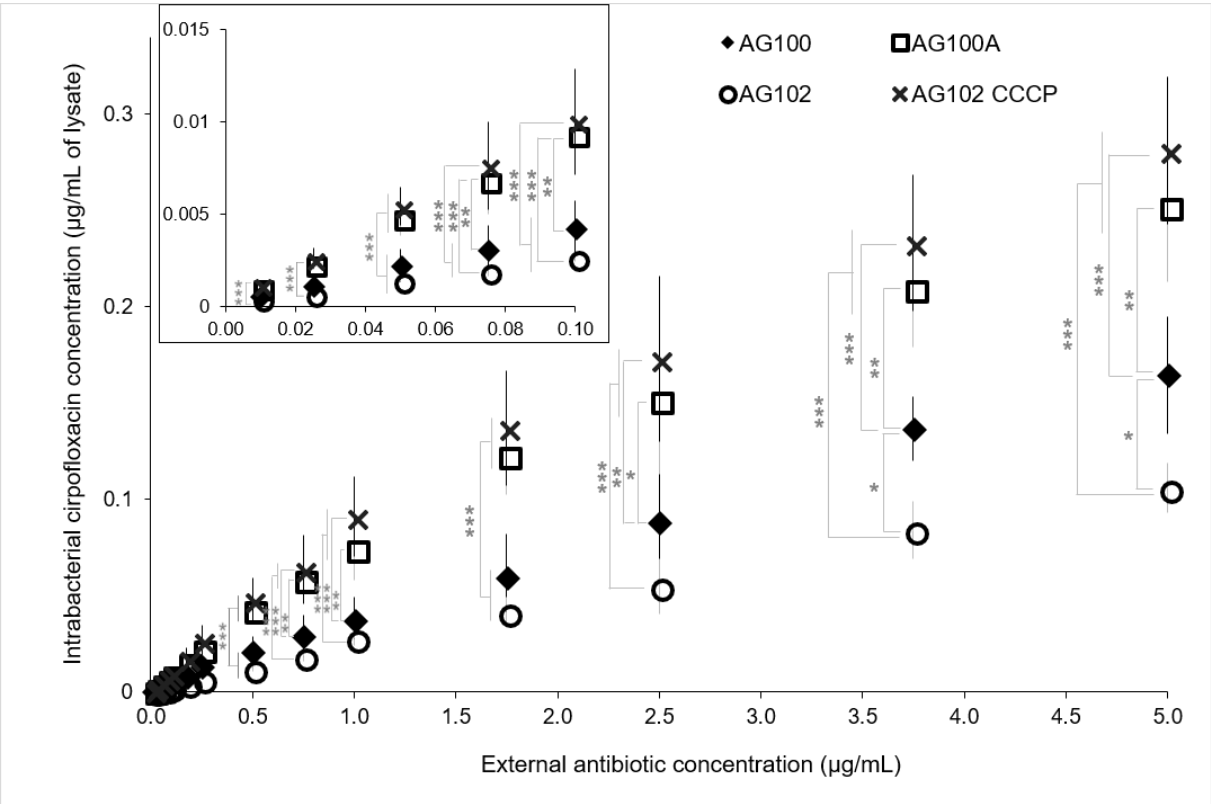
Figure 3. Amount of intrabacterial concentrations of ciprofloxacin and fleroxacin measured by mass spectrometry in the AG100A strain (AcrAB-).



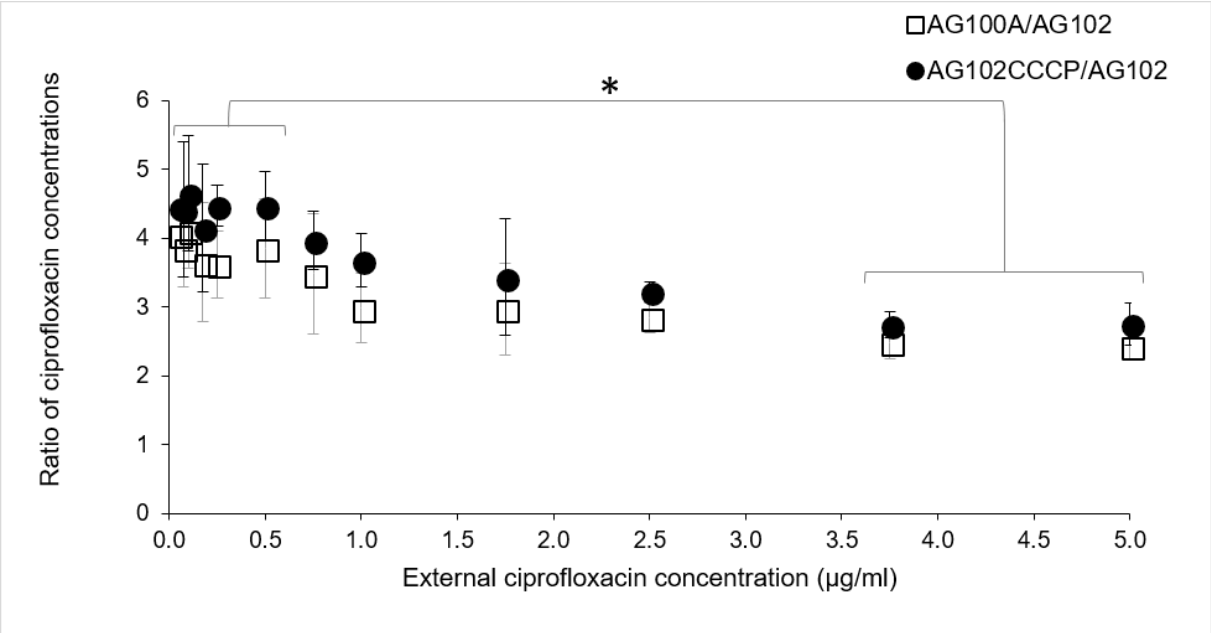
Increasing concentrations of ciprofloxacin (squares) and fleroxacin (circles) were accumulated for 10 min in AG100A (AcrAB-) cells. The concentrations accumulated in the various strains were measured by mass spectrometry. The symbols with bars (standard deviations) correspond to measurements carried out in triplicate.

Figure 4. Accumulation and concentration effect of ciprofloxacin measured by mass spectrometry in the studied strains expressing various efflux levels.



Increasing ciprofloxacin concentrations were accumulated for 10 min in AG100 (AcrAB+, black diamonds), AG100A (AcrAB-, white squares), AG102 (AcrAB++, white circles) and AG102 cells incubated in the presence of CCCP (10 µM) (crosses). The concentrations accumulated in the various strains were measured by mass spectrometry. The columns with bars (standard deviations) correspond to measurements of 3 independent experiments, each experiment being carried out in triplicate. The insert presents an enlargement for small concentrations. ANOVA and Tukey's post-Hoc tests were performed to determine differences between the various strains (n=9). ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$. Data normality was checked by the Shapiro-Wilk test and homogeneity of variances was checked by the Fligner-Killeen test.

Figure 5. Determination of ciprofloxacin accumulation ratios in *E. coli* strains expressing various efflux levels measured by mass spectrometry.



Increasing ciprofloxacin concentrations were accumulated for 10 min in AG100A (AcrAB-), AG102 (AcrAB++) and AG102 cells incubated in the presence of CCCP (10 µM). Two ratios of ciprofloxacin accumulation were calculated to characterize the efflux capability of the AcrAB efflux pump according to the external ciprofloxacin concentration: *[AG102+CCCP] / [AG102]* (black circles), where *[AG102+CCCP]* corresponds to the ciprofloxacin accumulated in AG102 (AcrAB++) in the presence of CCCP and *[AG102]* corresponds to the ciprofloxacin accumulated in the same strain in the absence of CCCP; similarly, *[AG100A] / [AG102]* (white squares), where *[AG100A]* corresponds to the ciprofloxacin accumulated in AG100A (AcrAB-) and *[AG102]* corresponds to the ciprofloxacin accumulated in AG102 (AcrAB++). The symbols with bars (standard deviations) correspond to measurements carried out in triplicate. The *[AG100A] / [AG102]* ratio is significantly higher at external concentrations of 0.05 or 0.1 µg/mL compared to external concentrations of 3.75 and 5 µg/mL

519 (ANOVA, $p<0.05$). Similarly, the *[AG102+CCCP] / [AG102]* ratio is significantly higher at
520 external concentrations of 0.05, 0.075, 0.1, 0.25 or 0.5 µg/mL compared to concentrations of
521 3.75 or 5 µg/mL (ANOVA, $p<0.05$). Data normality was checked by the Shapiro-Wilk test and
522 homogeneity of variances was checked by the Fligner-Killeen test.
523

524 **Table 1:** Mass spectrometry parameters used during analyses.

525

Ion source	**Ciprofloxacin**	**Fleroxacin**
Spray Voltage Positive Ion (V)	3500	3400
Sheath Gas (Arb)	40	45
Auxiliary Gas (Arb)	15	5
Sweep Gas (Arb)	2	1
Ion transfert Tube Temperature (°C)	350	350
Vaporizer Temperature (°C)	400	400
SRM	**Ciprofloxacin**	**Fleroxacin**
Collision Energy (V)	18	18
Dwell Time (ms)	50	50
RF lens (V)	75	77
Q1 Resolution (FWHM)	0.7	0.7
Q3 Resolution (FWHM)	0.7	0.7
CID Gas (mTorr)	1.5	1.5

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