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Abstract

Antibiotic (ATB) prescription in an intensive care unit (ICU) requires continuous monitoring of serum dosages due to the patient's pathophysiological condition. Dosing adjustment is necessary to achieve effective targeted concentrations. Since ICUs routinely use a large number of ATBs, global monitoring needs to be developed. In the present study, we developed a global analytical method for extracting, separating and quantifying the most widely used ATBs in ICUs: amoxicillin, piperacillin, cefazolin, cefepime, cefotaxime, ceftazidime, ceftolozane, ceftriaxone, ertapenem, meropenem, ciprofloxacin, moxifloxacin, levofloxacin, daptomycin, dalbavancin, linezolid and a beta-lactamase inhibitor: tazobactam. To guarantee the robustness of the quantification, we differentiated the 16 ATBs and the beta lactamase inhibitor into 4 pools (ATB1 to ATB4), taking into account prescription frequency in the ICU, the physicochemical properties and the calibration ranges of the ATBs selected. The whole ATB was then separated with two LC columns in reversed phase: Kinetex Polar-C18 100 Å and Polar-RP-80 synergy, in less than 6.5 min. Detection was carried out by electrospray in positive ion mode, by tandem mass spectrometry (LC-MS/MS. The four quantification methods were validated according to the European guidelines on bioanalytical method validation (EMEA guide), after determining the extraction yields, matrix effects, recovery, precision, accuracy, within-run precision and between-run precision. For all analyses, bias is <15% and is comparable to the literature and LOQs vary from 0.05 $mg.L^{-1}$ for ciprofloxacin to 1.00 $mg.L^{-1}$ for ceftriaxone and dalbavancin. The stability time of cefepime and piperacillin is 3 hrs and for the other ATBs 6 hrs in serum at room temperature. For long-term stability, freezing at -80°C guarantees 3 months of stability for ceftriaxone and dalbavancin and more than 6 months for the other ATBs.

Keywords

LC–MS/MS, Antibiotics, Therapeutic drug monitoring, Critical care medicine, Beta-lactamase, Sample preparation

Highlights

- Development of a monitoring method for 16 ATBs and beta lactamase inhibitor
- The 16 ATBs were grouped into 4 pools and quantified by specific methods
- Application of the method to 51 plasma samples from ICU patients
- Serum stability of Cefepime and Piperacillin is 3 hrs at room temperature
- The long-term stability of all ATBs is greater than 3 months at -80°C

1. Introduction

In intensive care units (ICUs), infection is a major problem due mainly to nosocomial infections. Many antibiotics from different families are then administered to treat patients. The various drug dosage regimens applied to hospitalized patients require precise monitoring of the antibiotic concentrations in plasma [1]. Thus, accurate and precise quantification of antibiotics in plasma is needed in order to avoid underexposure or overexposure of patients, which could lead to inefficacy and bacterial resistance or toxicity, respectively. This is further compounded by the frequent pathophysiological changes observed in critically ill patients, which lead to modifications of antibiotic pharmacokinetics (PK) [2–4]. Nowadays, the majority of analytical methods used to perform therapeutic drug monitoring (TDM) are dedicated to the homogeneous chemical family of ATBs (e.g. β-lactams [5–8], fluoroquinolones [9,10], oxazolidinones [11]). These analytical methods are essentially liquid chromatography techniques with UV detection. The methods involve specific sample preparation for each class of ATBs and do not allow rapid monitoring. Moreover, large-scale studies have shown that 16% of patients in ICUs have effective plasma concentrations below

75 the pharmacokinetic/pharmacodynamic (PK/PD) targets [12]. Thus, there is a need for precise
76 therapeutic drug monitoring (TDM) of antibiotics in plasma in order to individualize the
77 treatment regimen [1,13,14].

78 The limit of these analytical methods is found in the specific wavelengths used for each
79 therapeutic class, with UV quantification and dedicated sample treatment for specific
80 chemical families of ATBs. These methods are developed for only one chemical class, so they
81 are time-consuming and not adapted to ICU specifications. Developments in mass
82 spectrometry and tandem mass spectrometry have been published for the analysis of β-
83 lactams [15][16], peptides [17] and multi-class components [18], showing the possibility of
84 developing a comprehensive method, from extraction to separation, and optimizing the
85 detection parameters.

86 A few methods have been developed to ensure monitoring of a large panel of ATBs
87 commonly used in the ICU and are based on detection by high resolution mass spectrometry
88 (HRMS), a system that is rarely used in hospitals [19]. To take of all these constraints into
89 account, the present study focuses on developing an analytical methodology applied to
90 quantifying of 16 antibiotics and 1 beta-lactamase inhibitor. This methodology uses four
91 quantification methods based on reversed phase separation with tandem mass spectrometry
92 detection for monitoring the most commonly used ATBs in our ICU: two penicillins:
93 amoxicillin and piperacillin; six cephalosporins: cefazolin, cefepime, cefotaxime, ceftazidime,
94 ceftolozane and ceftriaxone; two carbapenems: ertapenem and meropenem; a beta-lactamase
95 inhibitor: tazobactam; three fluoroquinolones: ciprofloxacin, moxifloxacin and levofloxacin; a
96 glycopeptide: daptomycin; a lipoglycopeptide: dalbavancin; and an oxazolidinone: linezolid.
97 In order to take into account the different physicochemical properties of the analytes, the
98 specific concentration ranges for each ATB and the priorities for application in relation to the

frequency of administration for treated patients, four differentiated extraction and quantification methods were developed.

2. Material and methods

2.1 Chemicals

Amoxicillin (CAS-N. 61336-70-7 – purity 99.6%), piperacillin sodium salt (CAS-N. 59703-84-3 – purity 93.8%), cefazolin sodium salt (CAS-N. 27164-46-1– purity 97.7%), cefotaxime sodium (CAS-N. 64485-93-4 – purity 100%), ceftazidime hydrate (CAS-N. 120618-65-7– purity 98.4%), ceftriaxone disodium salt hemi (heptahydrate) (CAS-N. 104376-79-6 – purity 99.6%), meropenem trihydrate (CAS-N. 119478-56-7 – purity 99.1%), tazobactam sodium salt (CAS-N. 89785-84-2 – purity 92.5%), ciprofloxacin (CAS-N. 85721-33-1 – purity 99.5%), moxifloxacin hydrochloride (CAS-N. 186826-86-8 – purity 100%), levofloxacin (CAS-N. 100986-85-4 – purity 99.6%), dalbavancin (CAS-N. 171500-79-1 – purity 92.6%) and linezolid (CAS-N. 165800-03-3 – purity 99.5%) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). $^{2}H_{4}$-amoxicillin (CAS- 61336-70-7 – purity 96%), $^{2}H_{5}$-piperacillin (CAS-59703-84-3 – purity 95%), $^{13}C_{2}$ ^{15}N-cefazolin sodium salt (CAS-27164-46-1 – purity 98%), cefepime dihydrochloride monohydrate (CAS-N. 123171-59-5 – purity 95%), $^{2}H_{3}$-Cefepime sulfate (CAS- 123171-59-5 – purity 92%), $^{2}H_{3}$-cefotaxime (CAS-64485-93-4 – purity 95%), $^{2}H_{5}$-ceftazidime (CAS- 120618-65-7 – purity 90%), $^{2}H_{3}$-ceftriaxone disodium salt (CAS-N.1132650-38-4 – purity 96%), $^{2}H_{6}$-meropenem (CAS-119478-56-7 – purity 97%), $^{15}N_{3}$-tazobactam sodium salt (CAS- 89785-84-2 – purity 98%), $^{2}H_{8}$-ciprofloxacin (CAS-N.1130050-35-9 – purity 96%), daptomycin (CAS-N. 103060-53-3 – purity 98%) and $^{2}H_{3}$-linezolid (CAS-N.1127120-38-0 – purity 99.5%) were purchased from LGC (Molsheim, France). Ceftolozane trifluoroacetate salt (CAS-N.1628046-32-1 – purity

98.7%), $^{15}N_2$ $^{2}H_4$-ceftolozane trifluoroacetate salt (CAS-N.1628046-32-1 – purity 98.1%), ertapenem sodium salt (CAS-N.153773-82-1 – purity 92.7%), $^{2}H_4$-ertapenem sodium salt (CAS-N.153773-82-1 – purity 90.6%), $^{2}H_5$-moxifloxacin trifluoroacetate salt (CAS-N.1092356-43-8 – purity 99.7%), ^{13}C $^{2}H_3$-levofloxacin (CAS-N.1261398-33-7-– purity 99%), $^{2}H_6$-dalbavancin trifluoroacetate salt (CAS-N.1588823-86-2 – purity 95.3%) and $^{2}H_5$-daptomycin trifluoroacetate salt (CAS-N.13565796-56-0 – purity 97.3%) were purchased from ALSACHIM (Illkirch Graffenstaden, France). LC-MS-grade formic acid, acetonitrile and methanol were supplied by Honeywell-Fluka (Illkirch, France), isopropanol was purchased from VWR (Fontenay-sous-Bois, France) and water (UHPLC-MS quality) came from Scharlau (Barcelona, Spain).

2.2 Biological samples

Blank drug-free serum was provided by the French blood establishment. Clinical samples were collected as part of standard care from patients hospitalized in an ICU at Toulouse University Hospital between 01/01/2020 and 30/06/2021. This retrospective cohort study was approved by the Toulouse University Hospital review board (registration number: RnIPH 2021-78; CNIL number: 2206723 v 0).

2.3 Sample preparation

In order to overcome stability and weighing problems, two types of standard stock solutions and quality control for each molecule were weighed by two different manipulators and frozen at -80°C. One was used to prepare calibration ranges and the other for the preparation of the controls. Before use, they were tested by several injections at the same concentration in our analytical system and compared to give a difference of less than 5%. Each marked antibiotic used as an internal standard was prepared under the same

147 solubilization conditions and stored at -80°C (Table S4). The antibiotics used had to reach
148 different areas of therapeutic concentrations and the range amplitudes were adapted for each
149 mixture from ATB1 to ATB4, while three levels of quality control were prepared to cover the
150 full amplitudes of the different calibration ranges (Table S5). In order to reproduce a
151 representative patient sample, we were required not to exceed a ratio of 1/10 or 1 volume of
152 overload of stock solution and 9 volumes of matrix (serum, plasma) [19]. Low quality control
153 was prepared at 3 or 4 times the low quantification limit, with average control between 30%
154 and 60% of calibration ranges, and high control between 75% and 85% of calibration ranges
155 (Table S5).

156 Given the characteristics of solubility, the low and high quantification limits to be
157 reached, the large number of these molecules, and our choice not to exceed a ratio of 1/10 or 1
158 volume of stock solution overload and 9 matrix volumes (serum and plasma), it was very
159 difficult to monitor all the molecules in a single extraction procedure. To take into account the
160 differences in physicochemical properties related in particular to differences in the solubilities
161 of analytes, the ranges of concentrations sought and sensitivity, the 16 ATBs and the beta-
162 lactamase inhibitor were extracted differentially into 4 groups ATB1, ATB2, ATB3 and
163 ATB4 following 4 specific protocols. Each group is organized according to the frequency of
164 requests for monitoring ATBs in the Toulouse ICU and the physicochemical properties of the
165 16 ATBs and beta lactamase inhibitor. The ATB1 pool was built with ciprofloxacin,
166 tazobactam, amoxicillin, ceftolozane, cefepime, ceftazidime, piperacillin and cefazolin; the
167 ATB2 group contained cefotaxime, daptomycin, levofloxacin, linezolid and moxifloxacin;
168 ATB3 was built with ertapenem and meropenem; ATB4 contained the last 2 molecules
169 ceftriaxone and dalbavancin.

170 Sample pretreatment of all biological samples was carried out in 2 steps. In the first step,
171 the proteins were precipitated. Depending on the pool of ATBs, precipitation was carried out

with a mixture of Methanol with 0.1% formic acid for ATB1 to ATB3. For ATB4 precipitation was carried out with a mixture of acetonitrile and water (50/50) (vol./vol.) with 0.1% formic acid. The samples were then centrifuged. In a second step dilution with water with 0.1% formic acid was applied at differentiated ratios for ATB1 to ATB3. For ATB4 no dilution is carried out. All internal standards were contained in the precipitation solvent added in the first step.

For ATB1 to ATB3 protocols, the molecules were extracted and diluted with the same solvent mixture composed of Methanol / 0.1% formic acid and water / 0.1% formic acid, respectively. A simple deproteinization and adapted dilution of 1/39 for ATB1, 1/65 for ATB2 and 1/91 for ATB3 were used. For the ATB 4 group, we opted for a much milder precipitation.

ATB1 group: ATB1 internal standard stock solutions: $^{15}N_2$, $^{2}H_4$-Ceftolozane, $^{2}H_4$-Amoxicillin, $^{2}H_8$-Ciprofloxacin, $^{13}C_2$, $^{15}N_1$-Cefazolin, $^{2}H_3$-Cefepime, $^{2}H_5$-Ceftazidime, $^{2}H_5$-Piperacillin and $^{13}C_2$, $^{15}N_1$ Tazobactam were mixed extemporaneously and diluted in methanol with 0.1% (vol.) formic acid to 417, 208, 56, 278, 417, 556, 556 and 208 µg/L respectively. 300 µL of the stock solution of ATB1 internal standards was mixed with 25 µL of serum samples, standards or controls, vortexed 1 min and centrifuged at 10,000 g for 10 min. 200 µL of supernatant was recovered, diluted with 400 µL of 0.1% (vol.) formic acid in water, vortexed, transferred into autosampler vials and 2 µL was used for injection.

ATB2 group: ATB2 internal standard stock solutions: $^{2}H_3$-Cefotaxime, ^{13}C, $^{2}H_3$-Levofloxacin, $^{2}H_3$-Linezolid, $^{13}C_2$, $^{2}H_5$-Moxifloxacin and $^{2}H_5$-Daptomycin were mixed extemporaneously and diluted in methanol with 0.1% (vol.) formic acid to 1000, 325, 250, 500 and 2500 µg/L respectively. 240 µL of the stock solution of ATB2 internal standards were mixed with 20 µL of serum samples, standards or controls, vortexed 1 min and

centrifuged at 10,000 g for 10 min. 150 µL of supernatant were recovered, diluted with 600 µL of 0.1% (vol.) formic acid in water, vortexed, transferred into autosampler vials and 2 µL were used for injection.

ATB3 group: ATB3 internal standard stock solutions: $^{2}H_{6}$-Meropenem and $^{2}H_{4}$-Ertapenem were mixed extemporaneously and diluted in methanol with 0.1% (vol.) formic acid to 501 µg/L. 240 µL of the stock solution of ATB3 internal standards were mixed with 20 µL of serum samples, standards or controls, vortexed 1 min and centrifuged at 10,000 g for 10 min. 100 µL of supernatant were recovered, diluted with 600 µL of 0.1% formic acid (vol.) in water, vortexed, transferred into autosampler vials and 2 µL were used for injection.

ATB4 group: ATB4 internal standard stock solutions: $^{2}H_{3}$-Ceftriaxone and $^{2}H_{6}$-Dalbavancin were mixed extemporaneously and diluted in 0.1% (vol.) formic acid in water / 0.1% (vol.) formic acid in acetonitrile (50/50) (vol./vol.) to 1250 and 2400 µg/L respectively. 250 µL of the stock solution of ATB4 internal standards were mixed with 20 µL of serum samples, standards or controls, vortexed 1 min and centrifuged at 10,000 g for 10 min. 100 µL of supernatant were recovered and transferred into autosampler vials and 2 µL were used for injection.

2.4 Instrumentation and analytical conditions

The analyses were carried out by HPLC consisting of 2 high-pressure binary pumps (LC20ADXR) that can support up to 660 bars, an injector (SIL-20AXR) and a multi-channel column oven (6-way) (CTO-20AC) from Shimadzu (Marne la Vallée, France), coupled with a triple quadrupole mass spectrometer (Sciex API 4500) from ABSCIEX (Villebon-sur-Yvette, France). Given the concentrations to be achieved, the physical and chemical characteristics, and stability, 3 separation methods were developed to measure 16 molecules on a Kinetex

220 Polar-C18 100 Å 2.1 x 100 mm, 2.6 µm column from Phenomenex (Le Pecq, France) and a
221 method using a Synergi Polar-RP-80 Å 2 x 50 mm, 5 µm column from Phenomenex for the
222 two remaining molecules.

223 The mobile phase was composed of ultrapure water added with 0.1% formic acid (solvent A)
224 and acetonitrile added with 0.1% formic acid (solvent B), in order to obtain a pH of the
225 mobile phase below the lowest pKa of all the ATBs studied (Table S1). Solvents A and B
226 were delivered using dedicated gradients (Table S2), followed by an equilibration step for the
227 column before the next injection. The analysis times were respectively 6 min, 6.5 min, 5 min
228 and 4 min for each ATB pool. ATB1, ATB2 and ATB3 were separated on Kinetex 2.6 µm
229 Polar-C18 100 Å 100 x 2.1mm and ATB4 on Synergy 4 µm Polar-RP 80 Å 50 x 2 mm.

230 Given strong demand for analyses at our laboratory and thanks to our instrumentation
231 consisting of a column selector, our strategy was to minimize mobile phase changes for a
232 sequence overnight. Four different ATB groups (ATB1 to ATB4) were created to respond to
233 optimized separation, as well as to obtain concentration levels suitable for therapeutic
234 monitoring while having the chromatographic conditions best suited in terms of separation
235 and peak qualities (shape, asymmetry). Our choice was therefore based on 2 columns meeting
236 these criteria.

237 The LC/MS/MS conditions were set as follows: electrospray ionization (ESI) in positive
238 mode, spray voltage: 4500 V, gas pressure (nitrogen): curtain gas: 35 psi, ion source gas 1: 10
239 psi, ions source gas 2: 60 psi, collision gas: 10 psi, capillary temperature: 450 °C. All specific
240 parameters were set as described in Table S3.
241

242 2.5 Analytical method validation

Analytical validation was carried out according to the guidelines of the European Medicines Agency (EMEA) [20], indicating the set parameters that we had to test with the acceptability criteria that we had to meet to prove the robustness of our method before it goes into production. The selectivity of the method was studied by verifying its ability to quantify a reference element (here ATBs) without interference from other reference elements or endogenous compounds present in the matrix. It was tested using the appropriate blank matrix from at least six different sources. The signal of any interfering peak in each blank matrix should be less than 20.0% of the signal obtained at the LOQ for the reference element and 5.0% of the signal obtained for the internal standard.

The isotopic contribution was studied by verifying that no signal from the labeled internal standard (IS) interfered with the ATB to be quantified and vice versa. A first blank matrix was overloaded with the reference element to the concentration of the highest point of the range without IS, a second with the IS without the reference element (P0), and finally a third with the reference element to the LOQ without IS. The peak area obtained at the concentration of the highest point of the range and at the retention time of the IS should be less than 5.0% of the area of the peak of the IS obtained in the P0. The peak area observed in the P0 and the retention time of the reference element should be less than 20.0% of the peak area of the reference element obtained at the LOQ.

Carry-over was studied by verifying that no signal from the highly concentrated reference element (or its EI) from a previous injection would contaminate a blank matrix. The same blank was injected 3 times in a row after the highest standard of a calibration range. The peak signal interfering in each blank matrix should be less than 20.0% of the signal obtained at the LOQ for the reference element and 5.0% of the signal obtained for the IS without the reference element (P0).

The influence of the anticoagulant agent was measured by comparing the quantification of a serum sample with a heparinized plasma with the same concentration. Sample spikes were performed extemporaneously in triplicate with the low internal quality control (IQC) and high IQC level on our validation matrix (serum) as well as on lithium heparin plasma. The accuracy criteria were calculated relative to the concentration determination of IQC serum in triplicate. Accuracy and precision were to be less than 15%. A dilution study had to be validated beforehand in order to be able to measure samples that were higher than the last point of range. The dilution was done with the same matrix used for the overloads and the volume of the final test take had to correspond exactly to that of the method. A sample was prepared so that six extractions could be made. Its concentration is twice the highest point of the range. During analysis, it was diluted to 1/10. Accuracy and precision were to be less than 15%.

In initial validation, the global measurement uncertainty (GMU) was calculated as a percentage corresponding to the dispersion of values assigned to the measurements of our IQC (low, medium, high) during the between-run precision study. It is estimated in initial validation from eq. 1:

$$GMU = \frac{(\left|\overline{X} - Xref\right| + 2 * \sigma)}{Xref} * 100$$

Where x_{ref}: target value, $\overline{x}$: average value, σ: standard deviation, GMU: Global Measurement Uncertainty

Then, this value will be regularly updated by incorporating the results of external quality control (EQC) obtained within the year by inter-laboratory controls.

A calibration curve was used to establish a relationship between the response of the instrument and the concentration of the analyte. The calibration curves included six levels of

concentrations, a blank (matrix not overloaded with analyte or internal standard), and a P0 (matrix not overloaded with analyte but overloaded with internal standard). The P0 result was not included in the calibration curve. During validation, this was redone every day for five days and had to include the same points from one day to the next. The regression model was a linear model with a 1/X weighting and the regression coefficient had to be >0.995. The values of the first point and the last point of the range were not supposed to be excluded from the calculation of the calibration curve equation. Recalculated concentrations were expected to show a maximum accuracy of ± 20.0% for the first point of range and ± 15% for the other points.

The limit of quantification (LOQ) was the smallest amount of ATB that could be quantified with defined accuracy and precision. It corresponded to the first point of the calibration range with accuracy and precision of less than 20%. The upper limit of quantification (ULOQ), was the highest calibration standard.

The mathematical calculation compared with the measurements made showed us that the estimate gave a good reflection of reality.

The within-run precision concerned appreciating intra-series variability. For all three levels of controls, the samples were measured within a series with an extemporaneous calibration.

Precision is expressed as the coefficient of variation (CV) of peak areas from eq. 4.

$$(CV)\% = \frac{Standard\ Deviation}{Average} * 100$$

Accuracy describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage or 'bias') from eq. 5.

313 $$Bias = \frac{m - v}{v} * 100$$

314

315 Between-run precision consisted of appreciating inter-series variability (CV, eq. 4) with
316 the notion of time or even analyst or equipment (estimation of robustness). Five sets of
317 analyses were performed over five different days, each with an extemporaneous calibration,
318 and each level of IQC was extracted in duplicate. The mean values for each range point were
319 calculated over all five series. Each point had to show a precision and accuracy within the
320 limit of 15% (20% for the LOQ). The precision of the slopes of the calibration curves should
321 not exceed 15% with a regression coefficient >0.995. In each series, the accuracy of the CIQs
322 had to be within the 15% limit. For each concentration level, the average accuracy obtained
323 had to be less than 15% as well as the precision calculated from the ten IQCs. All the
324 quantitative methods developed on the 2 LC columns were validated according to these
325 criteria.

326 2.6 Extraction efficiency and ionization loss (matrix effect in LC-MS-MS)

327 The matrix effect, determined as previously described for quantitative bioanalytical
328 methods [21], corresponds to the potential interference in the ionization of molecules in the
329 source and may increase or decrease ionization efficiency, thus altering the device's signal.
330 Three parameters were studied:
331 The matrix effect, by comparing the signal (X) obtained from a blank extracted and then
332 overloaded with the reference element, with the signal of a pure solution (100).

333 $$Matrix\ effect = \frac{X}{100}$$

334 The extraction efficiency, by comparing the signal (Y) obtained from a sample overloaded
335 with the reference element and then extracted, with the signal (X) obtained from a blank
336 extracted and then overloaded with the reference element.

$$Extraction\ efficiency = \frac{X}{Y}$$

The recovery rate, by comparing the signal (Y) obtained from a sample overloaded with the reference element and then extracted, with the signal of a pure solution (100).

$$Recovery\ Rate = (Extraction\ efficiency * Matrix\ effect) = \frac{Y}{100} = \frac{Y}{X} * \frac{X}{100}$$

The three parameters were calculated in triplicate for each of the three levels of control and for the internal standard. The extraction efficiency and matrix effect were expected to be less than 15% (CV) for each level of concentration on the analyses. A matrix effect greater than 100% reflects an increase in ionization, while an effect less than 100% is the expression of an ion suppression phenomenon.

2.7 Stability

The stability of an ATB in a biological matrix depends on its chemical properties, matrix and storage conditions. Different stability conditions were tested to cover the different cases of pre-dosing storage.

2.7.1 Stability in whole blood

Overloads were carried out extemporaneously in triplicate at the level of low IQC and high IQC on our validation matrix (serum) as well as on whole blood taken on lithium heparin and extract (H0). The overloaded samples were stored for 24 hours at 4°C and then extracted in triplicate. The accuracy criteria were calculated based on the concentration of serum IQC (H0). Accuracy and precision were to be less than 15%.

2.7.2 Short-term stability

Overloads were performed extemporaneously in triplicate at the level of low IQC and high IQC on our validation matrix (serum) and extract (H0). Overloaded samples were stored for 3 hours and 6 hours at room temperature, for 24 hours, 48 hours and 72 hours at 4°C and then extracted in triplicate. The accuracy criteria were calculated based on the concentration of serum IQC (H0). Accuracy and precision were to be less than 15%.

2.7.3 Long-term stability

Overloads were performed extemporaneously in triplicate at the level of low IQC and high IQC on our validation matrix (serum) and extract (H0). Overloaded samples were stored for 1 month, 3 months, 6 months and 12 months at -20°C and -80°C and then extracted in triplicate. The accuracy criteria were calculated based on the concentration of serum IQC (H0). Accuracy and precision were to be less than 15%.

2.7.4 Freeze-thaw stability

Overloads were performed extemporaneously in triplicate at the level of low IQC and high IQC on our validation matrix (serum) and extract (H0). The overloaded samples were frozen for at least 24 hours and then completely thawed at room temperature and finally refrozen for a period of 24 hours (1st cycle). The second and third cycles were carried out in the same way. At the end of the third cycle, they were extracted in triplicate. The accuracy criteria were calculated based on the concentration of serum IQC (H0). Accuracy and precision were to be less than 15%.

2.7.5 Injector stability

Overloads were performed extemporaneously in triplicate at the level of low IQC and high IQC on our validation matrix (serum) and extract (H0). The extracts were stored for 24 hours at room temperature in the vials on the injector and then re-injected in triplicate. The

accuracy criteria were calculated based on the concentration of serum IQC (H0). Accuracy and precision were to be less than 15%.

2.7.6 Analysis of clinical samples

These analytical methods are now validated and used daily to determine antibiotic concentrations in plasma from patients at Toulouse University Hospital and nearby care facilities. As a proof of concept, we thus analyzed antibiotic concentrations determined for ICU patients. We chose to focus on cefepime for two reasons. On the one hand, cefepime has a narrow therapeutic index and a well-documented link between drug exposure and efficacy/neurotoxicity, and on the other hand it is by far the most requested antibiotic. We selected patients treated by continuous cefepime perfusion. Blood was collected on heparinized collection tubes when concentration reached the steady state. After arrival at the laboratory, samples were centrifuged at 3,500 rpm for 10 min and stored at -20°C before analysis. Measured concentrations were compared with guidelines published by two French working groups (i.e. the French Society of Anesthesia and Intensive Care Medicine (SFAR) and the French Society of Pharmacology and Therapeutics (SFPT)) [22].

3. Results and discussion

3.1 Chromatographic optimization

All the ATBs chosen for this study were in perfect harmony with the daily administration practiced in our intensive care units. In this period of COVID 19, many of these molecules had been administered and required rapid dosing to ensure optimum efficacy. The goal was to obtain selective methods that were quick and easy to implement with all specific classes of antibiotics (penicillins, cephalosporins, carbapenems, fluoroquinolones, etc.). All of the ATBs

studied contained specific chemical structures with very different physicochemical properties, as shown in Fig. S1 (supplementary data). The values of the solubility product constant (Ksp), as well as the octanol/water partition coefficients of each molecule, made it possible to choose the solvents best suited to optimal dissolution (Table S1). The solubilization properties of the analytes, described in Table S1, do not allow the use of a single generic method for the 17 molecules. We decided to develop four different quantification methods, grouping all 16 ATBs and 1 beta lactamase inhibitor into four pools, taking into account the frequency of requests for analysis of each molecule and their physicochemical properties. The compositions of the four ATB pools were mainly based on clinical priorities and practices: thus, the ATB1 pool was built to include the most prescribed antibiotic drugs in our hospital (ciprofloxacin, tazobactam, amoxicillin, ceftolozane, cefepime, ceftazidime, piperacillin and cefazolin). Overall, this pool represents more than 80% of antibiotic dosing demands from clinical departments. While most of the remaining antibiotics were then included in the ATB2 pool, some of them were isolated due to logistical and analytical constraints. The ATB3 pool included carbapenems (meropenem and ertapenem), which display poor stability in biological fluids [23] and therefore must be prioritized for analysis. The ATB4 pool, including daptomycin and ceftriaxone, had to be developed with different chromatographic conditions due to the poor performances of the initial conditions in terms of separation and peak aspect.

To mix a maximum of compounds, the concentrations to be reached for stock solutions had to be high. We therefore adapted the concentrations in relation to the high limits of linearity to be reached and established the antibiotic pools according to these different constraints. Typically, the upper limits of quantification for piperacillin and cefazolin were set at 40 $mg.L^{-1}$ and serum samples were diluted to quantitate the analytes in the linear domain (Table S4, supplementary data). During method development, we controlled the absence of

interference peaks with the ATBs studied in order to promote selectivity. Moreover, all ATBs had an isotopic contribution <20%.

Due to the complex structure of all the antibiotics studied, with negative or very high log P, we favored columns capable of performing hydrophilic and hydrophobic interactions and with the ability to work in 100% aqueous phase with maximum latitude in optimizing the elution gradient. The Kinetex Polar C18 column gave the best separation and peak shape results for ATB1 to ATB3 pools. However, it was not possible to obtain correct peaks with this column for ceftriaxone, probably due to the dioxotriazide group with this polar graft [19]. Better results were observed on a Synergi Polar-RP column, however, with an ether bonded phenyl phase and with a hydrophilic endcapping giving a satisfactory peak shape quality. We therefore developed the ceftriaxone method with this column, associated with dalbavancin, which does not pose any particular problems. The chromatographic separations of each pool was optimized using the iterative software Osiris®. The latter provided us with good predictions with the best separations (RS > 1.5 and 2<K<10), while having a short analysis time. However, it was not always possible for certain molecules (ceftolozane, cefepime, amoxicillin, tazobactam) (Table S1, supplementary data) with low values of octanol/water partition coefficients, to obtain a correct separation with resolution >1.5 (Fig S2, supplementary data), in less than 3 minutes of elution time, but in no way interfering with quantification. On the other hand, the ATB2 to ATB4 pools gave good separations (Fig S2, supplementary data). Similar problems of co-elution and separation with low resolution were obtained for these same classes of ATBs and beta lactamase inhibitor on semi-porous columns, with particle sizes equivalent to that used in our study, using buffered eluents. Amoxicillin, tazobactam and cefepime were also the polar compounds that show the least selectivity [24].

For the point linked to the MS transitions, ATBs and beta lactamase inhibitor are small molecules with molecular masses of less than 700 g/mol, except for daptomycin (1620.7 g/mol) and dalbavancin (1816.7 g/mol). Only one transition was followed, due to the high dose given to patients in ICU and the comparison made between the analytical result and the dose administered. For low masses, the mass spectrometer followed simply charged transitions protonated into the ESI source, and for the high masses, it followed doubly-charged protonated transitions. MRM transition tracking is listed on Table S3 (supplementary data).

3.2 Sample preparation

The results of the extraction yields, matrix effects and recovery rates for the sets of molecules are presented in Table 1. The very wide range of hydrophobicity in the ATBs, determined by log P at between -6.17 and 3.8 (Table S1) for the ATBs studied, led us to develop a method of extraction as simple as possible in terms of the number of steps and types of solvents used. The difficulty was to best adapt the matrix volumes collected for each group, on the one hand, and the composition and volume of the dilution reagent of the supernatant after precipitation, on the other hand, in order to respect the low and high quantification limits. As the amplitudes of the ranges were sometimes very large, we had to be able to correctly quantify very low levels of antibiotics, but also very high levels without risking saturating the detector. The matrix proteins of the first three ATB groups were precipitated with a mixture of methanol and formic acid at 0.1% and then the supernatant was diluted with the same solvent. For the ATB 4 group, we opted for a much gentler precipitation to avoid problems of a low recovery rate due to the adsorption of dalbavancin on proteins [24].

Ceftolozane, ceftazidime, levofloxacin and cefepime had ionization gains of 225, 174, 158 and 154%, respectively. The matrix effect is important for these 4 ATBs, which are polar,

476 with log P values of -6.17, -1.60, -0.40, -0.37, respectively (Table S1, supplementary data).
477 Lefeuvre et al. found values of >100% for ceftazidime, levofloxacin and cefepime, but of
478 <110%, using high resolution mass spectrometry and XIC mode for quantification, but no
479 information was given on the extraction efficiency and extraction yield [19]. Rigo-Bonnin et
480 al. developed a method showing a lower matrix effect (108%) for the quantification of
481 ceftolozane, in tandem mass spectrometry, but separating only this ATB and tazobactam [25].
482 For levofloxacin, a lower matrix effect was determined by Fang et al. in mouse plasma (94%),
483 with strong precipitation conditions, unusable with all ATBs [26]. The percentages of other
484 ATBs ranged from 90% to 130%, except for dalbavancin, which has a very significant
485 ionization loss at 20%.

486 Extraction yields were >65% except for ceftolozane (41%) and levofloxacin (65%). The
487 extraction yield was lower for ceftolozane than for the other ATBs, but for the method
488 developed in this study, it was higher than that found by Putnam et al. (20%), which used a
489 precipitation of serum proteins using 90% acetonitrile [27]. In the method developed by
490 Putnam et al. the very low extraction yield is observed as a very low matrix effect (96%).
491 Finally, recovery for all ATBs had always been between 64% and 138%, except for
492 dalbavancin at 20%, despite mild deproteinization, with precision on the three levels of
493 concentration (7%) <15%. However, the internal standards have always corrected this
494 characteristic phenomenon of the sometimes consequential LC-MS-MS. Precision measured
495 for extraction efficiency, matrix effect and recovery is <15% for all ATBs, except for
496 ceftolozane, cefepim, levofloxacin, linezolid, and moxifloxacin linked to the higher values for
497 matrix effects (Table 1).

498 Table 1: Extraction yields, matrix effects and recovery obtained with the 16 ATBs and 1
499 beta-lactamase with the differentiated quantification methods ATB1 to ATB4.

Antibiotics	Extraction efficiency		Matrix effect		Recovery	
	Rate	Precision	Rate	Precision	Rate	Precision
	%	%	%	%	%	%
			ATB 1			
Ceftolozane	41	11	225	22	92	19
Cefepime	89	13	154	16	137	19
Amoxicillin	92	8	105	8	96	8
Tazobactam	91	7	91	7	92	6
Ceftazidime	70	6	174	9	122	8
Ciprofloxacin	93	15	122	15	110	12
Cefazolin	88	5	105	5	92	5
Piperacillin	86	15	106	15	90	7
			ATB 2			
Cefotaxime	68	9	90	3	64	10
Daptomycin	88	10	94	7	82	11
Levofloxacin	65	20	158	25	107	40
Linezolid	80	9	99	13	80	18
Moxifloxacin	76	15	130	20	100	27
			ATB 3			
Ertapenem	68	4	127	7	87	3
Meropenem	81	5	130	2	105	5
			ATB 4			
Ceftriaxone	102	3	120	4	122	5
Dalbavancin	99	4	20	7	20	7

500

3.3 Method validation

3.3.1 Carry-over

Ertapenem and daptomycin had a significant memory effect of 169% and 30% requiring, after each CQI and depending on the patient, 3 injections of 10 µL of phase rinse (H2O/methanol/acetonitrile/isopropanol 25-25-25-25). The other ATBs had no memory effect phenomena.

3.3.2 Precision and accuracy

The analytical validation results are summarized in Table 2. For all analyses, bias is <15%. Ciprofloxacin has the largest bias observed in inter-day between-run precision (-11.7%) and piperacillin has the largest bias observed for intra-day within-run precision (-13.3%). For these same molecules, Lefeuvre and al. [19] observed lower biases, but ceftriaxone was analyzed with a high intra-day bias (14.1%), while for our study we observe a low value of bias (<2.8%) for this ATB. The relative standard deviation was acceptable for all ATBs. The highest values were observed for piperacillin (11.8%) for between-run precision and for daptomycin (7.8%) for within-run precision, which is consistent with the literature. The specifications to be met were reached in terms of within-run precision, between-run precision and accuracy.

Table 2: Accuracy, Within-run and between-run precision results.

	Accuracy and within-run precision (Intra-day)			Accuracy and between-run precision (Inter-day)		
	Low QC *	Medium QC *	High QC *	Low QC*	Medium QC *	High QC *

	Molecules	Bias %	CV %	Bias %	CV %	Bias %	CV %	Bias %	CV %	Bias %	CV %	Bias %	CV %
ATB1	Ciprofloxacin	-11.1	7.1	4.8	5.1	11.0	3.3	-11.7	5.5	-2.2	7.6	3.3	7.1
	Tazobactam	-6.3	4.9	-5.3	6.7	-0.5	7.1	-10.0	4.9	-9.9	5.1	-6.8	5.7
	Amoxicillin	-8.8	4.8	-10.0	3.8	-6.7	2.7	-3.3	6.8	-0.6	9.3	-1.4	10.0
	Ceftolozane	-8.2	5.5	-11.0	4.0	-7.8	3.1	-8.6	5.9	-3.7	10.3	-0.6	8.7
	Cefepime	-11.8	6.0	-8.4	3.7	-1.6	4.8	-7.6	6.5	-3.9	8.8	-2.3	7.9
	Ceftazidime	-1.8	2.6	-3.5	5.1	-1.1	6.8	0.2	6.7	-3.9	5.7	-2.0	8.9
	Piperacillin	-11.3	2.4	-13.3	2.6	-12.5	2.7	-3.5	8.6	-2.7	9.1	0.9	11.8
	Cefazolin	-9.0	3.8	-4.2	6.2	-3.9	4.7	-9.9	3.8	-4.3	4.0	-5.7	4.4
ATB2	Levofloxacin	4.7	3.6	7.4	2.8	7.4	2.8	-1.5	5.2	2.0	4.8	2.9	4.1
	Ofloxacin	-11.6	1.9	-2.1	7.6	-13.0	0.9	-8.6	8.0	-9.5	5.8	-10.4	4.8
	Moxifloxacin	-11.6	4.2	-5.7	1.6	-5.1	1.1	-7.5	7.7	-2.4	5.6	-2.1	7.0
	Linezolid	-10.5	3.9	-7.4	2.0	-4.8	2.4	-10.1	5.5	-11.4	2.8	-8.7	3.0
	Cefotaxime	-6.8	5.4	0.5	5.5	-3.8	2.5	-7.9	4.0	-0.4	4.1	-6.4	3.5
	Daptomycin	8.6	7.8	3.4	6.1	6.1	4.9	6.3	10.4	0.1	5.5	3.1	6.6
ATB3	Meropenem	4.3	2.2	3.1	3.7	2.3	2.1	4.2	2.7	4.3	3.2	4.0	2.8
	Ertapenem	-5.7	5.8	-6.8	2.8	-7.7	1.6	-2.9	6.3	-4.9	5.7	-5.3	7.1
ATB4	Ceftriaxone	-0.7	1.4	0.8	1.6	-2.8	1.7	2.5	4.1	2.7	4.4	-1.9	4.1
	Dalbavancin	1.9	2.5	5.6	0.6	3.7	1.7	3.6	5.2	6.0	1.7	3.2	1.9

* Low QC, Medium QC and High QC are given for each ATBs in Table S5.

3.3.3 Limit of quantification (LOQ)

The LOQs were studied and all corresponded to the first point of the ATB calibrations, for which accuracy and precision were measured with a standard deviation of <20% (Table 3). These LOQs were adapted to each low value of the ATBs sought in patients' serums. They ranged from 0.05 $mg.L^{-1}$ for ciprofloxacin to 1 $mg.L^{-1}$ for ceftriaxone and dalbavancin. Higher values were expected in serums from ICU patients with pathophysiological features, which are integrated into the final dosage by the systematic dilution applied to all samples (1/10), in order to meet the guidelines of specific ICU therapeutic practices [22]. For most literature studies, LOQs are set at 0.5 mg.L-1 or 1 mg.L-1 for ATBs. [16,19].

The ULOQs were also studied and all corresponded to the last point of the ATB calibrations, for which accuracy and precision were measured with a standard deviation of

534 <15% (Table 3). They ranged from 2.5 mg.L^{-1} for ciprofloxacin to 100 mg.L^{-1} for
535 Cefotaxime, Daptomycin, Meropenem and Ertapenem.

536 By differentiating the quantification into 4 pools, it is possible to monitor of 16 ATBs and
537 the beta lactamase inhibitor using an MS/MS detection based on a single transition, with
538 within-run precision, between-run precision and accuracy close to those using an HRMS
539 detection [19]. If dilution of the serum is an additional step, it is however mandatory, because
540 of the high doses administered. The robustness of this step is correctly evaluated for High QC
541 values, with CVs<15% (Table 2). The validation of these 4 quantification methods shows
542 performances equivalent to those obtained for the monitoring of 15 ATBs in plasma
543 developed by Lefeuvre et al. [19], in which all compounds were detected by HRMS and
544 quantified with a linear regression between 0.5 and 32 mg/L (R^2 > 0.998). The 4 pools ATB1,
545 ATB2, ATB3 and ATB4 show a quantification with a linear regression adapted to each ATB
546 as presented in Table 3. The LOQ and ULOQ are modulated according to the prescriptions
547 and target values [12]. In all the ranges sought, the linearity of the calibration is validated
548 (R^2>0.998) (Table 3). The slopes of the calibration lines are in the same order of magnitude as
549 those obtained with an HRMS detection mode, although comparisons are limited, since the
550 internal standards used are not all identical or used at the same concentration [19]. The
551 advances obtained with this new methodology are the modulation of the calibration ranges
552 and the validation of the robustness of the 4 extraction and quantification pools.

553 Table 3: Measured LOQ, ULOQ and linear regression parameters for the 16 ATBs and 1
554 beta-lactamase inhibitor.

555

	Measured LOQ (mg.L^{-1})	Measured ULOQ (mg.L^{-1})	Linear regression

	Molecules			Slope Intercept R^2
ATB1	Ciprofloxacin	0.05	2.5	y = 1.418x - 0.0853 (R^2 = 0.999)
	Tazobactam	0.10	5	y = 1.044x + 0.0935 (R^2 = 0.999)
	Amoxicillin	0.20	10	y = 0.540x + 0.0239 (R^2 = 0.999)
	Ceftolozane	0.40	20	y = 0.202x - 0.0009 (R^2 = 0.998)
	Cefepime	0.40	20	y = 0.189x + 0.0089 (R^2 = 0.999)
	Ceftazidime	0.40	20	y = 0.146x - 0.0010 (R^2 = 0.999)
	Piperacillin	0.80	40	y = 0.097x - 0.0019 (R^2 = 0.999)
	Cefazolin	0.80	40	y = 0.130x + 0.0168 (R^2 = 0.999)
ATB2	Levofloxacin	0.13	25	y = 0.290x + 0.0014 (R^2 = 0.999)
	Moxifloxacin	0.13	25	y = 0.267x - 0.0039 (R^2 = 0.999)
	Linezolid	0.13	25	y = 0.235x + 0.0150 (R^2 = 0.999)
	Cefotaxime	0.50	100	y = 0.076x + 0.0109 (R^2 = 0.998)
	Daptomycin	0.50	100	y = 0.144x + 0.0421 (R^2 = 0.999)
ATB3	Meropenem	0.20	100	y = 0.139x + 0.0023 (R^2 = 0.999)
	Ertapenem	0.20	100	y = 0.129x + 0.0041 (R^2 = 0.999)
ATB4	Ceftriaxone	1.00	50	y = 0.064x + 0.0109 (R^2 = 0.999)
	Dalbavancin	1.00	50	y = 0.044x + 0.0033 (R^2 = 0.999)

3.3.4 Anticoagulant, dilution, global measurement uncertainty and stability.

This accuracy and precision study first showed that heparinized tubes can be accepted for monitoring 16 ATBs and the beta lactamase inhibitor, bias and CV being <15% for the high and low QC values (Table 4). Global measurement uncertainty was small <30%, without reference in the literature.

Table 4: Lithium Heparin Validation Results, Dilution, and Global Uncertainty Calculation.

		Comparison of doped serum versus doped heparinized plasma				Validation of dilution		Global measurement uncertainty		
		Low QC		High QC		Dilution (1/10 ULOQ)		Low QC	Medium QC	High QC
	Molecules	Bias %	CV %	Bias %	CV %	Bias %	CV %	CV %	CV %	CV %
ATB1	Ciprofloxacin	0.5	4.9	-1.5	1.9	12.6	2.0	21.3	17.1	17.9
	Tazobactam	-13.4	1.2	-9.5	4.7	1.9	7.0	18.9	19.0	17.3
	Amoxicillin	-0.3	7.6	-7.7	0.3	6.0	4.1	16.4	19.1	20.0
	Ceftolozane	-8.3	9.6	-6.1	0.3	4.7	5.9	19.4	23.6	17.9
	Cefepime	-11.5	4.0	-9.3	0.3	9.8	3.2	19.6	20.8	17.7
	Ceftazidime	-9.3	4.8	-5.2	3.2	12.9	3.1	13.5	14.8	19.5
	Piperacillin	-8.9	3.7	-3.4	0.3	6.1	2.6	20.2	20.3	24.7
	Cefazolin	-6.1	5.0	-6.9	4.4	8.7	2.8	16.7	12.1	13.9

ATB2	Levofloxacin	10.4	0.8	4.4	1.8	12.0	2.5	11.8	11.7	11.4
	Ofloxacin	1.1	0.8	-0.6	1.2	2.5	-0.6	23.2	20.0	19.0
	Moxifloxacin	-5.5	6.2	1.0	3.0	8.9	5.4	21.8	13.4	16.9
	Linezolid	-1.6	3.7	4.9	3.2	-7.8	3.3	20.0	16.3	14.2
	Cefotaxime	-8.1	3.2	3.4	2.3	8.7	3.6	15.2	8.6	13.0
	Daptomycin	-6.8	2.8	0.0	4.8	7.8	4.8	28.3	11.2	16.7
ATB3	Meropenem	0.6	2.2	1.9	0.2	1.3	5.3	9.7	10.9	9.9
	Ertapenem	7.9	1.1	1.3	1.9	3.5	4.6	15.0	15.8	18.8
ATB4	Ceftriaxone	3.1	0.9	-3.9	2.0	11.5	1.9	11.0	11.7	10.0
	Dalbavancin	4.8	7.9	-2.6	3.9	3.4	5.4	14.4	9.5	7.1

A study of the stability of all the antibiotics was carried out and is shown in Table 5.

Table 5: Short-term, long-term, autosampler and freeze-thaw stability results in serum and whole blood, for the 4 differentiated extraction and quantification ATBs pools (ATB1 to ATB4).

		Stability						
		Short-term			Long-term		Autosampler	Freeze-thaw
	Spiked sample	Room temperature	4°C		-20°C	-80°C	Room temperature	Room temperature / -80°C
		Serum (hours)	Whole blood (hours)	Serum (days)	Serum (months)	Serum (months)	Extract (hours)	Serum (number)
ATB 1	Ciprofloxacin	6	24	2	12	12	24	3
	Tazobactam	6	24	2	1	6	24	3
	Amoxicillin	6	24	5	1	6	24	3
	Ceftolozane	6	24	2	6	6	24	3
	Cefepime	3	24	2	1	6	24	3
	Ceftazidime	6	24	5	3	6	24	3
	Piperacillin	3	24	1	1	6	24	3
	Cefazolin	6	24	2	12	12	24	3
ATB 2	Levofloxacin	6	24	3	6 (test 12 months in progress)	6 (test 12 months in progress)	24	3
	Ofloxacin	6	24	3	6 (test 12 months in progress)	6 (test 12 months in progress)	24	3

	Moxifloxacin	6	24	3	6 (test 12 months in progress)	6 (test 12 months in progress)	24	3
	Linezolid	6	24	3	6 (test 12 months in progress)	6 (test 12 months in progress)	24	3
	Cefotaxime	6	QC Low not stable 24 for QC High	3	3	6 (test 12 months in progress)	24	3
	Daptomycin	6	24	3	6 (test 12 months in progress)	6 (test 12 months in progress)	24	3
ATB 3	Meropenem	6	not stable 24 H	1	not stable 1 M	6 (test 12 months in progress)	not stable 24 H	3
	Ertapenem	6	24	2	not stable 1 M	6 (test 12 months in progress)	not stable 24 H	3
ATB 4	Ceftriaxone	6	24	3	3 (test 6 and 12 months in progress)	3 (test 6 and 12 months in progress)	24	3
	Dalbavancin	6	24	3	3 (test 6 and 12 months in progress)	3 (test 6 and 12 months in progress)	24	3

The stability study shows that some ATBs and the beta lactamase inhibitor cannot be quantified after 3 hours of stacking of samples at room temperature, such as cefepime and piperacillin. For piperacillin, Mortensen et al. showed 6-hr stability at room temperature for human plasma treated with EDTA [23]. The stability of these sensitive ATBs and the beta lactamase inhibitor is always low (1 month), after freezing at -20°C, but above 6 months, if the stacking is carried out at -80°C. The remaining stability durations are consistent with those given in the literature [15,17–19]. On total blood, we find 2 ATBs that are not stable over a duration of 24 hrs at 4°C: cefotaxime for the low QC and meropenem.

3.4 Application to clinical samples.

Cefepime was the most prescribed antibiotic for patients in ICUs, representing around 80% of total antibiotic requests. As such, we chose to focus our study on this antibiotic, found in the ATB1 pool. Cefepime levels were measured at steady state in patients treated with continuous infusion of the antibiotic. Daily dose of cefepime varied between 2000 mg and 8000 mg

according to identified bacteria, infection site and the patients renal function. In total, 50 cefepime plasma concentrations were measured with the ATB1 quantification method. Their distribution is represented in Fig.1.

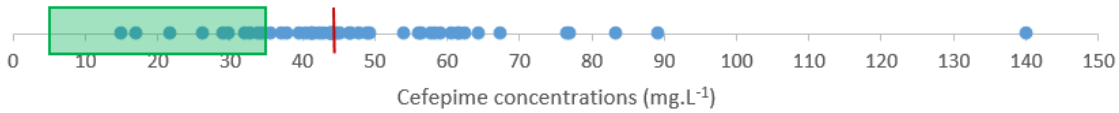

Fig.1. Distribution of measured concentrations of cefepime in ICU patients (in green: recommended concentration window from the SFAR-SFPT consensus; in red: median value of measured concentrations).

The recommended concentrations window is 5 to 35 $mg.L^{-1}$ for unknown bacterial infections in the case of a continuous infusion [22]. While no underexposure was detected, indicating sufficient antibiotic efficacy, around 75% of measured concentrations were above the recommended concentrations range. This point is particularly important as our results highlight an increased risk of toxicity, and more precisely neurotoxicity [28]. This can be easily explained by the pharmacokinetic behavior of the drugs administered to ICU patients. Indeed, this population tends to have damaged renal functions, leading to a decrease in cefepime elimination. Monitoring cefepime concentrations is thus essential to optimize patient care.

4. Conclusion

In this work, we developed the analysis of 16 ATBs from different classes and one beta-lactamase inhibitor, using a simple protein precipitation method and grouping the ATBs into 4 pools, to rapidly respond to requests for assays, prioritizing the most requested ATBs in the ATB1 pool. For the 4 quantification methods evaluated in this study, sampling was limited to 20 µL of serum, whereas conventional methods use 50 µL to 100 µL. These quantification

methods allow wide spectrum monitoring from the most commonly administered ATBs (ATB1) to the last molecules grouped in ATB3 and ATB4. The separation methods use the same solvents, to simplify the column change needed to quantify all 17 molecules, while LC-MS-MS technology enabled us to optimize the analysis time as much as possible (extraction, injection, integration and validation). Performance was evaluated and validated according to the EMEA guidelines and demonstrated a robust, easy-to-implement method. As we had also seen widely varying stabilities for the different ATBs, we improved sample flow and storage upon receipt in the laboratory. In addition, as this technology allowed for extremely low volume (a few µL), the amount of sample was reduced. This was particularly interesting for pediatric samples and pharmacokinetic studies.

Developing this new method was motivated by an optimization of patient care. In this context, therapeutic drug monitoring for antibiotics has to be performed 7 days a week to adjust drug dosage as quickly as possible. Our goal has been achieved, since our strategy was to extend this methodology to transfer our old methods and optimize them as best we could with our more efficient instruments.

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