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Simultaneous determination of 8 beta-lactams and linezolid by an ultra-performance liquid chromatography method with UV detection and cross-validation with a commercial immunoassay for the quantification of linezolid

D. Fage *, G. Deprez, B. Fontaine, F. Wolff, F. Cotton

Clinical Chemistry Department – LHUB-ULB, Brussels, Belgium

A B S T R A C T

Linezolid and beta-lactams are anti-infective drugs frequently used in intensive care unit patients. Critical illness could induce alterations of pharmacokinetic parameters due to changes in the distribution, the metabolism and the elimination process. Therapeutic drug monitoring (TDM) is therefore recommended to prevent mainly under-dosing of beta-lactams or hematological and neurological toxicities of linezolid. In Multi-or Extensively-Drugs Resistant-Tuberculosis Bacteria, the regimen could include linezolid with meropenem and amoxicillin/clavulanate justifying the development of a method allowing their simultaneous quantification.

The aim of this work was to develop an in-house ultra-performance liquid chromatography method with UV detection (UHPLC-PDA) allowing the simultaneous determination of 8 beta-lactams (amoxicillin, aztreonam, cefepime, ceftazidime, ceftriaxone, cefuroxime, meropenem and piperacillin) and linezolid and to cross-validate the linezolid quantification with a new commercial immunoassay (ARK kit) tested on a Cobas analyzer. The main advantages of the immunoassay are a 24/24 h random access assay which is fully automated and results provided within 2 h.

The interference due to potential co-administrated drugs was evaluated on both methods. The preanalytical factors (type of matrix, stability) for linezolid were also investigated. The influence of hemolysis, icteria or lipemia on the spectroscopic detection of the immunoassay was assessed. The analytical performances were evaluated using the accuracy profiles approach with acceptance limits fixed at ±30%. Seventy patient samples were measured using both methods.

No cross-reaction with the tested anti-infective drugs as well as no influence of hemolysis, lipemia, icteria were observed. The linezolid concentration could be measured on heparinized plasma or serum without a significant difference and remained stable for at least 72h at 4°C.The UHPLC-PDA method performed well in the analytical range investigated (0.25–50 mg/L for meropenem, 0.75–50 mg/L for linezolid and 1–200 mg/L for other beta-lactams) with an intermediate precision and a relative bias below 7.6 and 7.7%, respectively. The analytical range of the immunoassay was narrower, from 0.85 to 18.5 mg/L. The precision and relative bias were lower than 8.1% and 4.2%, respectively. Results obtained on clinical samples showed an acceptable difference between methods with a mean bias of -1.8% [95% confidence interval: -5.2% – 1.6%].

To conclude, both methods showed acceptable performance to perform TDM of linezolid considering the therapeutic through target of 2-8 mg/L. The choice of the method should be made according to the degree of emergency of the response required and the field of application justifying or not the simultaneous quantification of beta-lactams and linezolid.

1. Introduction

Beta-lactams are hydrophilic compounds with moderate (30–70%) to low (<30%) protein binding, low volume of distribution and major renal clearance. They are extensively used to treat Gram-negative infections. Intensive care unit (ICU) patients are highly subject to inadequate dosing due to altered pharmacokinetics or external factors such as continuous renal replacement therapy. Indeed, ICU patients may manifest increased apparent volume of distribution caused by capillary leak syndrome, impaired drug distribution and elimination due to end-organ dysfunction and augmented renal clearance explained by increased renal blood flow and glomerular hyperfiltration. All these physiopathological modifications explain the high pharmacokinetic inter-patient variability observed in critically ill patients and the significant risk of under-dosing hydrophilic compounds. The evidence of misdosing beta-lactams are well-known and the recommendation are to perform routinely the therapeutic drug monitoring (TDM) of beta-lactams in this particular population [1–4].

Linezolid (LZD) is a synthetic anti-infective drug belonging to the oxazolidinone family. It is mainly used for its bacteriostatic effect against Gram-positive cocci resistant to standard treatment (methicillin-resistant *S. aureus,* vancomycin-resistant enterococci and cephalosporin-

* Corresponding author.
E-mail address: david.fage@lhub-ulb.be (D. Fage).

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resistant *S. pneumoniae*) [5] and against Multi- or Extensively-Drugs Resistant-Tuberculosis Bacteria (MDR-TB or XDR-TB) [6]. In the recent literature, extensive evidence supports the clinical utility of TDM of linezolid for ICU [7–9] and tuberculosis patients [6,10,11]. The physiological alterations observed with critical illness and altered pharmacokinetic parameters were described above [3,4]. For MDR-TB or XDR-TB, the therapy has a minimal length of 6 months and could include LZD in association with meropenem (MEM) and amoxicillin (AMX)/clavulanate in a regimen of five effective drugs [12–15]. But, side hematological effects (thrombopenia, leucopenia and anemia) as well as peripheral neuropathy can occur after 2 weeks of LZD therapy [16,17] and are strongly associated with trough serum levels above 20 mg/L [18]. The work of Cattaneo et al. showed that an early TDM could prevent long-term overexposure of patients, a situation at high risk of adverse events appearance. Indeed, LZD has a narrow trough therapeutic range of 2–8 mg/L [16]. A concentration above 8 mg/L inhibits the synthesis of platelet precursor cells. Moreover, the drug has a wide inter-individual variability for a same recommended posology (600 mg twice daily). Some covariates such as kidney function, co-medications, body weight and age explain a part of the variability [16,18]. For all these reasons, the TDM of LZD is mandatory.

Many in-house published methods are available to quantify several beta-lactams or linezolid. Most of the recent assays to quantify a panel of antibiotics are based on a liquid chromatography coupled with tandem mass spectrometry [19–26] that require instruments that are not readily available in many laboratories. At the best of our knowledge, there is no available method on ultra-performance liquid chromatography (UHPLC) system coupled to a photodiode array detector (PDA) to simultaneously monitor the main beta-lactams subject to TDM (MEM, piperacilline [PIP], ceftazidime [CZA] and ceftriaxone [CFT]) [27] and LZD, which could be used in combined therapy with them. Therefore, the purpose of this work was to develop an UHPLC method allowing the simultaneous determination of 8 beta-lactams (AMX, aztreonam [AZT], cefepime [CEF], CZA, CFT, cefuroxime [CFX], MEM and PIP) and LZD and to cross-validate it with a new automated immunoassay for LZD determination from ARK Diagnostics.

2. Materials and methods

2.1. Reagents and chemicals

LZD was obtained from Pfizer (Brussels, Belgium). CEF and AZT were purchased from Bristol-Myers (Braine-l'Alleud, Belgium), MEM from AstraZeneca (Brussels, Belgium), AMX, CFX and CZA from GlaxoSmithKline (Wavre, Belgium), PIP from Wyeth Pharmaceuticals (Louvain-la-Neuve, Belgium) and CFT from Roche (Anderlecht, Belgium). Cefoperazone, used as internal standard (IS) was purchased from Sigma-Aldrich (Bornem, Belgium) and VWR (Leuven, Belgium), respectively. Acetonitrile (ACN) and methanol (MeOH) were obtained from Biosolve (Valkenswaard, The Netherlands) and orthophosphoric acid 85% from MerckMillipore (Brussels, Belgium). All reagents were analytical grade. Ultrapure water was obtained by means of a Milli-Q water purification system (MerckMillipore, Brussels, Belgium).

2.2. UHPLC method

The method was based on a previously published one [28] allowing the quantification of 6 beta-lactams (AZT, CEF, CZA, CFX, MEM and PIP) on an Agilent 1200 HPLC system (Agilent Technologies, Diegem, Belgium). The method was upgraded to an AQUITY UPLC-PDA system (Waters, Zellik, Belgium) equipped with a quaternary pump, a thermostated autosampler, a column oven and a photodiode array detector and allowed the separation and quantification of 8 beta-lactams (AMX, AZT, CEF, CZA, CFT, CFX, MEM and PIP) and LZD within 22 min.

Samples were thermostated at 8 °C and the separation was performed at 45 °C using an ACQUITY UPLC HSS T3 (Waters, Zellik, Belgium) column (2.1 mm × 100 mm) packed with 1.8 μm diameter particles and protected with a Assy Frit 0.2 μm for 2.1 μm (Waters, Zellik, Belgium). The volume of injection was 10 μL. The mobile phases consisting of 2% ACN and 0.6% (105 mmol/L) phosphate solution, pH 5 (MPA) and ACN (MPB) were delivered at a flow rate of 0.6 mL/min according to the following gradient: from 0 to 1.0 min the mobile phase contained 100% MPA; from 1.0 to 4.0 min, the percentage of MPB increased to 2.7%; from 4.0 to 11.5 min, MPB increased to 15%; from 11.5 to 13.5 min, MPB increased to 17.3%; from 13.5 to 15.5 min, MPB increased to 30.7% and was maintained at this percentage until 16.0 min; from 16.0 to 16.5 min, MPB increased to 100% and was maintained at this percentage until 18.5 min; from 18.5 to 18.7 min, the percentage of MPA returned to the initial conditions and was maintained at 100% until 22.0 min. The column eluent was monitored spectrophotometrically in the 205–400 nm range.

2.3. Automated immunoassay

The ARK Linezolid assay is a homogeneous immunoassay based on competition between drug in the sample and LZD labeled with recombinant enzyme glucose-6-phosphate dehydrogenase (G6PDH) for binding to the antibody reagent. As the LZD labeled binds antibody, the enzyme activity decreases. In the presence of drug from the specimen, the enzyme activity increases and is proportional to the drug concentration. The active enzyme converts the coenzyme nicotinamide adenine dinucleotide (NAD) to NADH that is measured spectrophotometrically as a rate of absorbance change at 340 and 415 nm.

The kit was installed according to the manufacturer recommendations on a Cobas c702 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The sample volume was 3 μL with a dead volume of 250 μL. The time of reaction was 5 min. The declared analytical range was 0.75–30.00 mg/L. The assay was calibrated with 6 points ARK Linezolid calibrators (0, 1, 2.5, 5, 15 and 30 mg/L). As there is no internationally recognized standard for LZD, ARK Linezolid calibrators are prepared by gravimetric dilution of high purity linezolid (traceable to HPLC) into a synthetic proteinaceous matrix free of linezolid.

2.4. Preparation of stock solutions, calibration and validation standards

Standard stock solutions of AMX, AZT, CEF, CZA, CFT, CFX, LZD, MEM, PIP and IS were prepared by dissolving powdered antibiotics in purified water at final concentrations of 40,000 mg/L for beta-lactams and 2000 mg/L for LZD. All solutions were stored at −80 °C and were stable at this temperature for at least 12 months (data not shown).

Calibration standards (CS) were prepared in a pool of drug-free sera at final concentrations of 0.25, 0.5, 1, 2, 10 and 50 mg/L for LZD and MEM and at final concentrations of 1, 2, 4, 8, 40 and 200 mg/L for AMX, AZT, CEF, CZA, CFT, CFX, and PIP according to the following protocol. Stock solutions were diluted in purified water at a concentration of 500 mg/L for LZD and MEM and 2000 mg/L for AMX, AZT, CEF, CZA, CFT, CFX, and PIP. Intermediate solutions were then prepared by diluting the mixed solution 500/2000 mg/L at concentrations of 2.5, 5.0, 10, 20, 100 and 500 mg/L for LZD and MEM and at concentrations of 10, 20, 40, 80, 400 and 2000 mg/L for AMX, AZT, CEF, CZA, CFT, CFX, and PIP. A pool of drug-free sera was finally spiked with intermediate solutions 10 fold more concentrated (constant ratio serum: intermediate aqueous solution of 9 : 1). Validation standards (VS) were prepared at the same concentrations for the UHPLC-PDA method and at the final concentrations of 0.5, 1, 2, 10 and 25 mg/L with a similar diluting protocol for the immunoassay.

Quality control samples were prepared by diluting stock solutions in a pool of drug-free sera to achieve final concentrations of 5 and 25 mg/L for MEM and LZD and of 20 and 100 mg/L for AMX, AZT, CEF, CZA, CFT, CFX and PIP.

2.5. Sample treatment

Blood samples of patients were collected without any anticoagulant, immediately chilled and centrifuged at 4 °C for 10 min at 4000 rpm. Sera were frozen at −80 °C until analysis. After thawing, the aliquots were vortex-mixed and then centrifuged at 4 °C for 10 min at 2000 g to clarify the supernatant.

For chromatography analysis, 50 µL of the IS working solution (200 mg/L) were added to 200 µL of sample. Controls, CS and VS were treated using the same pre-treatment protocol as the samples. Serum protein precipitation was performed by adding 800 µL of methanol. The mixture was vortex-mixed for 30 s and centrifuged at 4 °C for 10 min at 23,800 g. Eight hundred µL of supernatant were recovered and then evaporated under a gentle nitrogen stream. The residue was reconstituted with 200 µL of 2% ACN and 0.6% phosphate solution, pH 5 (MPA). The resulting solution was mixed for 30 s and centrifuged at 4 °C for 10 min at 23,800 g to eliminate potential residual proteins. The supernatant was transferred into a UHPLC vial (Waters, Zellik, Belgium) and 10 µL were injected into the UHPLC-PDA system.

For the immunoassay, no pretreatment was necessary.

2.6. Specificity

Interference studies were run accordingly to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations [29] with regard to anti-infective drugs frequently co-administered with beta-lactams and LZD: vancomycin, azithromycin, tobramycin, amikacin, clarithromycin, moxifloxacin, levofloxacin, ciprofloxacin, colistin, voriconazole, posaconazole, 11-hydroxy-itraconazole, fluconazole, rifampicine, isoniazide, ethambutol and pyrazinamide. For the immunoassay, the interference of beta-lactams (AMX, AZT, CEF, CZA, CFT, CFX, MEM and PIP) was also assessed.

An aqueous solution of the potential interfering substance was added to CS to yield a final concentration in the clinical range. Two levels of CS were used, one containing LZD and MEM at 2 mg/L and AMX, AZT, CEF, CZA, CFT, CFX, and PIP at 20 mg/L and one containing LZD and MEM at 2 mg/L and AMX, AZT, CEF, CZA, CFT, CFX, and PIP at 200 mg/L. The added volume of interfering compound solution never exceeded 10% of total volume to minimize the dilution of the CS. Vancomycin, azithromycin, clarithromycin were tested at a final concentration of 20 mg/L, rifampicine, isoniazide, ethambutol, moxifloxacin, levofloxacin and ciprofloxacin at a final concentration of 10 mg/L and pyrazinamide at 35 mg/L. Beta-lactams and antifungals were tested in a range of concentration between 1 and 200 mg/L and 0.1–10.0 mg/L, respectively. Interferences from tobramycin, amikacin and colistin were assessed at a final concentration of 2 mg/L. Paired CS containing the same dilution with Milli-Q water were analyzed simultaneously.

The interference of hemolysis, lipemia and icterus (serum indices) was evaluated for the immunoassay only. The serum indices are easily obtained by spectrophotometry on Cobas analyzers and provide a semi-quantitative measurement of hemolysis, lipemia or icterus in the samples. Influence of serum indices on ARK results was evaluated from 0 until 1000 for the hemolysis index, from 0 until 100 for the lipemia index and from 0 until 30 for the icteria index on a drug-free sera pool spiked with a LZD stock solution for a final concentration at 5 mg/L. The pool with serum indices at zero was defined as the paired sample.

All samples were analyzed in triplicate to minimize random error. The difference between mean concentrations measured in paired samples was defined as the bias. The decision of acceptability was based on a maximum bias of 10% (difference that could have a clinical impact according to the therapeutic range of LZD).

2.7. Extraction procedure

The relative recovery (%) was calculated in triplicate at two levels (5 and 25 mg/L for LZD and MEM; 5 and 200 mg/L for AMX, AZT, CEF, CZA, CFT, CFX, and PIP) by dividing the analyte peak area ratio obtained for extracted spiked sera by those obtained after extraction of water spiked at the same concentration.

The absolute recovery (%) was assessed in triplicate at two concentration levels (5 and 25 mg/L for LZD and MEM; 5 and 200 mg/L for AMX, AZT, CEF, CZA, CFT, CFX, and PIP) by dividing the analyte peak area ratio obtained for extracted spiked sera compared by those obtained on spiked MPA solutions at the same levels not subjected to the extraction procedure.

2.8. Type of sample

Serum and plasma matrices were evaluated for LZD measurement. Pools of sera and heparinized plasma were prepared with residual samples sent to the lab for routine biochemical and endocrinology analyses (patients not treated by LZD). The pools were analyzed with both ARK and UHPLC methods to confirm the absence of LZD. Spiked samples were prepared by diluting stock solutions in a pool of drug-free sera and heparinized plasma to achieve final antibiotic concentrations of 1, 2, 10 and 25 mg/L. All samples were analyzed in duplicate to minimize random error. The difference between median concentrations in plasma and serum was evaluated with a Wilcoxon-test performed with GraphPad Prism software, version 5.0 (San Diego, USA). A p value lower than 0.05 was considered statistically significant.

2.9. Stability

For short-term stability of LZD, spiked samples were prepared by diluting stock solutions in a pool of drug-free sera and plasma to achieve final antibiotic concentrations of 5 and 25 mg/L. The stability was tested at initial conditions, after 12 h on the bench and after 24, 48 and 72 h at 4 °C. The difference between the concentrations measured initially and at each tested condition was defined as the bias. The decision of acceptability was based on a maximum bias of 10% (difference that could have a clinical impact according to the therapeutic range of LZD).

For long-term stability of LZD, spiked samples were prepared by diluting stock solutions in a pool of drug-free sera and plasma to achieve final antibiotic concentrations of 5 and 25 mg/L. The aliquots were stored at −80 °C and analyzed once a month during 3 months.

2.10. Method validation

The accuracy profiles approach was used to validate the immunoassay and UHPLC-PDA methods [30–33]. The acceptance limits were fixed at ± 30% according to the regulatory requirements and the risk of having future results falling outside those limits was set at 5% [34]. CS were assessed at 6 different levels in triplicate during three consecutive days for the UHPLC-PDA method. The immunoassay was calibrated with ARK calibrators (0, 1, 2.5, 5, 15 and 30 mg/L). The VS were analyzed independently at different levels in quadruplicate during three consecutive days. Trueness, precision, accuracy, limit of quantification and linearity were reported for each method.

For the immunoassay, the intermediate precision was also assessed with ARK quality controls (2, 10 and 20 mg/L). Each level was analyzed two times per day, at different times, during 15 days.

2.11. Methods comparison

The comparison was performed on 70 clinical samples. A Bland-Altman analysis, performed with GraphPad Prism software, version 5.0 (San Diego, USA), was used to compare the results.

The samples were residual sera from biochemical routine analysis of patients treated by LZD. Two aliquots of sera per patient were frozen at −80 °C until analysis with immunoassay or UHPLC-PDA method. The paired aliquots were analyzed the same day with both methods. As this work was done retrospectively on residual human material to perform

assays comparison, the informed patient consent was not requested. Ethics approval (AK/10-06-41/3907) according to the declaration of Helsinki was obtained from the local ethic committee.

3. Results

3.1. UHPLC-PDA method

3.1.1. Chromatographic conditions

The absence of compounds co-eluting with AMX, AZT, CEF, CZA, CFT, CFX, MEM, PIP, LZD and IS in the pool of human drug-free sera was checked chromatographically.

Fig. 1 shows chromatograms of an extracted blank serum sample and an extracted calibration standard with a final antibiotic concentration of 100 mg/L of each compound, excepted for CFX, MEM and LZD (25 mg/L). Under the conditions described, the peaks corresponding to each compound were resolved with retention times of 4.39 (AZT), 4.85 (CEF), 5.45 (CZA), 6.25 (MEM), 6.92 (CFT), 9.43 (CFX), 9.87 (AMX), 13.55 (IS), 15.27 (LZD) and 16.13 min (PIP).

3.1.2. Specificity

None of the 17 antibiotics tested (vancomycin, azithromycin, tobramycin, amikacin, clarithromycin, moxifloxacin, levofloxacin, ciprofloxacin, colistin, voriconazole, posaconazole, 11-hydroxy-itraconazole, fluconazole, rifampicine, isoniazide, ethambutol and pyrazinamide) interfered with the chromatographic quantification of beta-lactams, LZD and IS. Indeed, the difference between the CS with and without potential interfering substances never exceeded the acceptable bias (data not shown).

3.1.3. Extraction procedure

The relative and absolute recoveries are summarized in Table 1. They were well reproducible at the concentrations tested. The absolute recovery obtained for the IS analyzed in triplicate was 73.8% (±1.0).

3.1.4. Type of sample

No significant difference was observed between heparinized plasma and serum matrices for LZD (p value = 0.1).

3.1.5. Stability

The short-term and long-term stabilities of beta-lactams were not investigated because they were already well described. It is stated that beta-lactams sampling must be immediately chilled, centrifuged at 4 °C and supernatant should be frozen at −80 °C for long-term storage (until 9 months) and analyzed within 8 h [35,36].

For short-term stability of LZD, no significant degradation was observed in plasma and serum at different conditions investigated (bias ≤5%). In contrast with beta-lactams, the serum of plasma with LZD could be kept at room temperature for 12 h and at 4 °C for 3 days. For long-term stability, no significant degradation was observed in plasma and serum at different conditions investigated (bias ≤5%).

Table 1
Mean of absolute and relative recovery obtained for antibiotics quantified at two levels of concentration.

Antibiotics	Concentration (mg/L)	RR (%) – SD	AR (%) – SD
Amoxicilline	5	80–4	82–3
	200	80–3	85–3
Aztreonam	5	76–4	76–3
	200	72–6	76–3
Cefepime	5	66–5	73–5
	200	67–5	70–3
Ceftazidime	5	73–5	76–4
	200	70–5	72–2
Ceftriaxone	5	82–5	83–3
	200	85–3	87–4
Cefuroxime	5	85–4	86–5
	200	76–5	78–5
Piperacilline	5	87–4	82–4
	200	75–5	77–2
Meropenem	5	89–7	76–3
	25	84–8	71–5
Linezolid	5	104–3	105–3
	25	105–3	102–4

3.1.6. Method validation

The trueness, expressed by the relative bias, the precision including the repeatability and intermediate precision, as well as the linearity results are shown in Table 2 for beta-lactams and in Table 3 for LZD. The antibiotic concentration was quantified with a known trueness and precision in the ranges of 0.25–50.00 mg/L for MEM, 0.75–50.00 mg/L for LZD and 1–200 mg/L for AMX, AZT, CEF, CZA, CFT, CFX, and PIP.

3.2. ARK method

3.2.1. Specificity

None of the 25 antibiotics tested (AMX, AZT, CEF, CZA, CFT, CFX, MEM,PIP, vancomycin, azithromycin, tobramycin, amikacin, clarithromycin, moxifloxacin, levofloxacin, ciprofloxacin, colistin, voriconazole, posaconazole, 11-hydroxy-itraconazole, fluconazole,

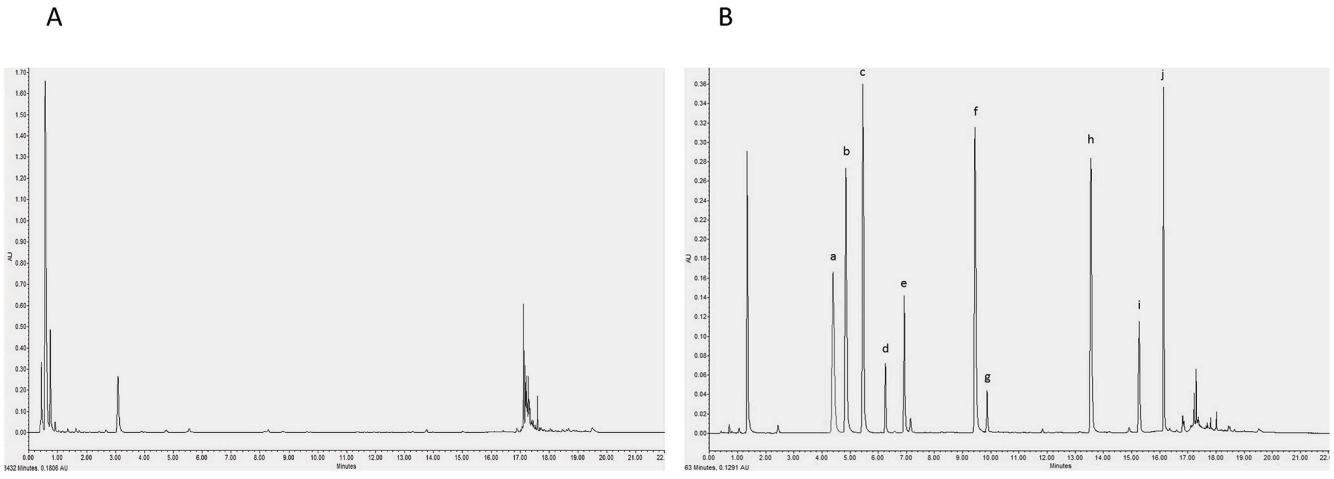

Fig. 1. Chromatograms of an extracted blank serum sample (A) and an extracted calibration standard containing a final antibiotic concentration of 100 mg/L of each compound, excepted for CFX, MEM and LZD (25 mg/L) (B). Aztreonam (a), cefepime (b), ceftazidime (c), meropenem (d), ceftriaxone (e), cefuroxime (f), amoxicilline(g), cefoperazone (h), linezolid (i) and piperacilline (j) were separated within 22 min and monitored spectrophotometrically in the 205–400 nm range.

Table 2
Criteria of analytical performance (trueness, precision, accuracy and linearity) obtained for beta-lactams measurements. Calibration curves were built using a quadratic regression weighted 1/X.

Validation criteria	Amoxicilline	Aztreonam	Cefepime	Ceftazidime	Ceftriaxone	Cefuroxime	Piperacilline	Validation criteria	Meropenem
Trueness	**Mean concentration (mg/L)/Relative bias (%)**							**Trueness**	
Concentration: 1 mg/L	1.0/3.0	1.0/3.4	1.0/2	1.1/7.7	1.0/2.2	1.0/2.5	1.0/-0.4	Concentration: 0.25 mg/L	0.25/-0.3
Concentration: 2 mg/L	–	2.0/2.1	2.0/0.6	2.1/3.1	2.0/2.6	2.0/0.7	2.0/0.8	Concentration: 0.5 mg/L	0.49/-2.7
Concentration: 4 mg/L	4.0/-1.0	4.0/0.1	4.0/-0.3	4.0/0.3	4.0/0.2	4.0/0.2	4.0/-0.8	Concentration: 1 mg/L	0.96/-3.6
Concentration: 8 mg/L	–	8.0/0.4	8.0/-0.1	8.0/0.3	8.0/0.1	8.0/0.2	8.0/-0.1	Concentration: 2 mg/L	1.91/-4.5
Concentration: 40 mg/L	40.0/0.1	40.3/0.8	40.5/1.2	40.6/1.6	40.0/0.9	40.0/1.1	40.1/0.1	Concentration: 10 mg/L	9.30/-7.0
Concentration: 200 mg/L	200.1/0.0	201.7/0.9	203.5/1.7	195.9/-2.1	199.6/-1.2	198.6/-2.2	198.2/-0.9	Concentration: 50 mg/L	48.00/-4.1
Precision	**CV[a] intermediate precision (%)**							**Precision**	
Concentration: 1 mg/L	5,0	2,8	2,0	2,3	2,1	2,0	2,5	Concentration: 0.25 mg/L	3,4
Concentration: 2 mg/L	–	1,8	1,9	2,7	1,7	1,9	1,9	Concentration: 0.5 mg/L	5,0
Concentration: 4 mg/L	2,3	1,9	2,4	5,1	2,1	2,4	2,8	Concentration: 1 mg/L	4,2
Concentration: 8 mg/L	–	2,1	2,3	4,3	3,2	2,3	3,4	Concentration: 2 mg/L	4,7
Concentration: 40 mg/L	2,7	2,2	2,4	2,7	2,5	2,2	2,3	Concentration: 10 mg/L	7,6
Concentration: 200 mg/L	1,7	1,7	2,2	1,8	1,9	1,7	2,7	Concentration: 50 mg/L	3,9
Accuracy	**Lower β-expectation limit (%)/Upper β-expectation limit (%)**							**Accuracy**	
Concentration: 1 mg/L	−8.9/15.0	−6.3/20.8	−7.0/9.3	−10.7/19.1	−7.3/19.8	−7.0/9.3	−5.2/16.0	Concentration: 0.25 mg/L	−8.2/15.1
Concentration: 2 mg/L	–	−5.1/7.4	−15.9/13.9	−11.8/10.8	−6.1/6.4	−15.9/13.9	−3.9/7.2	Concentration: 0.5 mg/L	−6.0/6.4
Concentration: 4 mg/L	−6.5/4.6	−12.0/19.7	−10.3/13.2	−4.1/10.0	−11.0/15.7	−10.3/13.2	−2.6/12.3	Concentration: 1 mg/L	−9.3/15.0
Concentration: 8 mg/L	–	−10.4/14.5	−10.2/10.6	−8.8/12.7	−10.4/14.5	−10.2/10.6	−6.6/12.0	Concentration: 2 mg/L	−6.5/10.3
Concentration: 40 mg/L	−6.5/6.6	−2.5/13.3	−5.7/17.1	−3.5/13.0	−2.5/13.3	−5.7/17.1	−0.7/12.8	Concentration: 10 mg/L	−1.3/12.3
Concentration: 200 mg/L	−4.0/41	−2.8/13.9	−3.7/19.5	−6.9/17.7	−2.8/13.9	−3.7/19.5	−2.0/15.8	Concentration: 50 mg/L	−9.1/15.9
Linearity	**Slope (95% confidence intervals)**							**Linearity**	
	1.006 (0.998–1.009)	1.009 (1.004–1.013)	1.018 (1.012–1.023)	0.979 (0.974–0.984)	1.011 (1.008–1.015)	1.018 (1.013–1.022)	0.991 (0.984–0.997)		0.959 (0.950–0.969)

[a] Coefficient of variation.

rifampicine, isoniazide, ethambutol and pyrazinamide) interfered with the immunoassay. Indeed, the difference between the CS at 2 and 10 mg/L and the ones spiked with potential interfering substances never exceeded the acceptable bias (data not shown).

No influence of hemolysis, lipemia and icteria was observed on the LZD quantification in the range of serum index investigated.

3.2.2. Type of sample

No significant difference was observed between heparinized plasma and serum matrices (p value = 0.1).

3.2.3. Stability

For short-term and long-term stability, no significant degradation was observed in plasma and serum at different conditions investigated (bias ≤7%).

3.2.4. Method validation

Results are shown in Table 3. The LZD concentration was quantified with a known trueness and precision in the ranges of 0.85–18.50 mg/L. The intermediate precision (CV %) assessed with ARK quality controls was 7.1%, 6.7% and 5.6% at concentrations of 2, 10 and 20 mg/L, respectively and were in agreement with imprecision obtained with VS (5.9% for 2 mg/L and 7.5% for 10 mg/L).

3.3. Methods comparison

Seventy clinical samples were collected for the methods comparison. Fifteen samples were below the lower limit of quantification (<0.85 mg/L) and eight were above the upper limit of quantification of the ARK LZD assay (>18.50 mg/L). So finally, 47 samples were included in the comparison.

The results showed a mean bias of −1.8% with a 95% confidence interval of [-5.2% – 1.6%] between both methods (Fig. 2.

4. Discussion

We developed an in-house UHPLC-PDA method for the simultaneous quantification of 8 beta-lactams and LZD commonly used to treat bloodstream infections caused by bacteria in ICU patients. The decision to develop this panel assay was guided by two aspects. Firstly, effective

Table 3
Criteria of analytical performance (trueness, precision, accuracy and linearity) obtained for LZD measurements. Calibration curves were built using a linear regression without weighing.

Validation criteria	Linezolid UHPLC-PDA	Linezolid ARK kit
Trueness	**Mean concentration (mg/L)/Relative bias (%)**	
Concentration: 0.25[b] mg/L	0.30/19.3	–
Concentration: 0.5[b] mg/L	0.57/13.2	0.67/33.3
Concentration: 1 mg/L	1.01/1.1	1.00/0.0
Concentration: 2 mg/L	1.92/-4.1	1.92/-4.2
Concentration: 10 mg/L	10.19/1.9	9.50/-0.5
Concentration: 25[b] mg/L	–	23.69/-5.2
Concentration: 50 mg/L	51.18/2.4	–
Precision	**CV[a] intermediate precision (%)**	
Concentration: 0.25 mg/L	48.4	–
Concentration: 0.5 mg/L	18.3	17.4
Concentration: 1 mg/L	5.8	8.1
Concentration: 2 mg/L	4.0	5.9
Concentration: 10 mg/L	3.5	7.5
Concentration: 25[b] mg/L	–	12.3
Concentration: 50 mg/L	4.8	–
Accuracy	**Lower/Upper β-expectation limit (%)**	
Concentration: 0.25 mg/L	−102.7/141.4	–
Concentration: 0.5 mg/L	−32.7/59.1	−8.4/75.1
Concentration: 1 mg/L	−13.4/15.6	−19.4/19.4
Concentration: 2 mg/L	−14.0/5.8	−18.6/10.2
Concentration: 10 mg/L	−6.5/10.3	−23.1/13.1
Concentration: 25[b] mg/L	–	−35.2/24.8
Concentration: 50 mg/L	−9.5/14.2	–
Linearity	**Slope (95% confidence intervals)**	
	1.024 (1.012–1.035)	0.943 (0.912–0.975)

[a] Coefficient of variation.
[b] Not acceptable: total error above the accuracy limits.

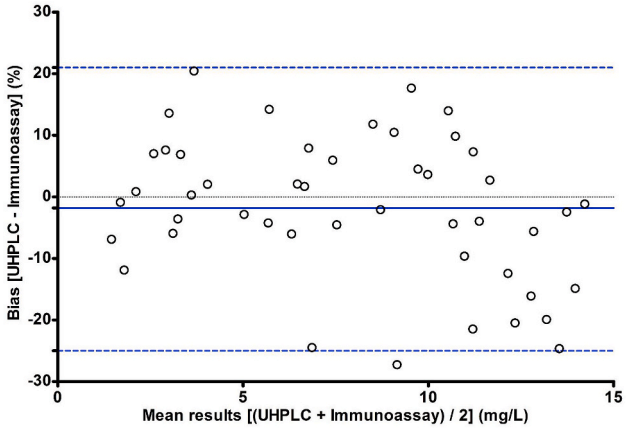

Fig. 2. Relative Bland-Altman comparison of LZD measurements between the UHPLC-PDA and the ARK Immunoassay method. The plain blue line represents the mean relative bias (−1.8%) and the dashed blue lines are the 95% limits of agreement [-22.5% - 27.0%]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

TDM for ICU patients requires results available on the same-day [1,2]. The development of an assay panel quantifying several of the most frequently used antibiotics in hospitalized patients was done to minimize the number of methods and samples needed. The use of the same analytical method represents an advantage in terms of turnaround time, decreasing costs when compared to several methods performed on different instruments and limiting the number and the sample volume, this point being critical with pediatric patients. Secondly, our lab is associated to the CHU Saint-Pierre where 250 to 300 tuberculosis patients are followed each year. So, the inclusion of LZD to a beta-lactams panel makes sense because the combination AMX/clavulanate/-MEM/LZD is a potential therapeutic regimen for MDR-TB or XDR-TB infection [12–15].

The advantages to perform the TDM of beta-lactams and LZD on a UHPLC system for ICU and tuberculosis patients are: (1) the availability of the instrument; (2) the high resolution limiting the risk of coelution with other drugs in these peculiar populations of patients treated with combinations of therapies; (3) the high sensitivity allowing the determination of each drugs with a low limit of quantification compatible with the minimal inhibitory concentration of the drug; (4) the short analysis time, the low solvent consumption and the rapidity of column equilibration between two injections are critical to perform TDM of a panel with a high throughput and a short turnaround time.

The process of solvent deproteinisation with methanol was chosen as it required a low sample volume, was technically easier and cheaper than solid-phase extraction and allowed the best recovery for beta-lactams [37]. The absolute recovery was excellent (≥70% for beta-lactams and around 100% for LZD). The sample volume was equivalent or slightly higher than other methods allowing the simultaneous determination of beta-lactams with UHPLC-UV system [36–38]. However, the LOQ of our method was lower (1 mg/L against 2 or 5 mg/L) and more adequate with the therapeutic target. For example, CEF and CZA have a clinical breakpoint defined by the European Committee on antimicrobial susceptibility testing (EUCAST) at 1 mg/L (*free*MIC). These two drugs are weakly bound to plasma proteins (17% for CEF and 10% for CZA), so a reasonable estimation could be *f*MIC = MIC. As, the minimal therapeutic target is a serum level superior at the MIC during 100% of the time, a method allowing a quantification of the MIC (1 mg/L) is preferable. The separation time was similar to other published methods. Commercial chromatographic methods are also available on the market. However, they allow the quantification of fewer molecules, sometimes with multiple chromatographic conditions. Other antimicrobial drugs (vancomycin, azithromycin, tobramycin, amikacin, clarithromycin, moxifloxacin, levofloxacin, ciprofloxacin, colistin, voriconazole, posaconazole, 11-hydroxy-itraconazole, fluconazole, rifampicine, isoniazide, ethambutol and pyrazinamide) commonly co-administrated with beta-lactams or LZD did not interfere.

The ARK LZD assay is a new immunoassay available since mid-2018 for automated clinical chemistry analyzers. The time of reaction is extremely short, about 5 min, depending on the analyzer. The volume of sample needed is similar between the chromatographic and the immunoassay. The quantification could be performed on serum or heparinized plasma. None of the 25 antibiotics tested (same as above plus AMX, AZT, CEF, CZA, CFT, CFX, MEM,PIP) interfered with the immunoassay. The advantages of this commercial method are a 24/24 h random access assay which is fully automated, results provided within 2 h, the possibility of quantify simultaneously multiple samples and no requirement of specialized personnel. In comparison, the chromatographic method can only be performed once or twice a day by batch, with a longer turnaround time mainly due to the extraction protocol, need training personnel and requires automated Liquid Handling equipment, an expensive instrument, to attempt the same level of automation, traceability and prevent human error inherent to a manual protocol extraction.

The UHPLC-PDA method had a wide quantification interval of 0.75–50.00 mg/L with an intermediate precision (CV%) ≤5.8% and a relative bias ≤4.1% for LZD determination while the immunoassay demonstrated a narrower analytical range of 0.85–18.50 mg/L with an intermediate precision (≤8.1%) and a relative bias (≤4.2%) quite similar. The accuracy profiles showed that the imprecision of the immunoassay increased with concentration and was not acceptable above 18.5 mg/L. The methods comparison showed an excellent concordance of results in the analytical range of the immunoassay with a non-significant mean bias of −1.8% [95% IC: -5.2% – 1.6%]. Only one result fell outside the 95% limits of agreement.

Castoldi et al. compared the same ARK LZD assay with their in-house HPLC-UV method [39]. Their chromatographic method based on an acidic protein precipitation required a higher sample volume (300 μL) to

a similar analytical range (0.4–48 mg/L). Their evaluation of the performances of the ARK LZD assay performed with QCs from ARK showed comparable results (intermediate precision (CV%) ≤6.9% and relative bias ≤6.6%). Their methods comparison also concluded that the concordance between both methods was acceptable but that the ARK assay was less accurate for LZD levels above 10 mg/L. This cut-off was obtained from the Bland-Altman comparison showing that nearly 80% of results falling outside the 95% limit of agreement had a LZD concentration exceeding 10 mg/L. The Youden index for best sensitivity/-specificity calculated on a receiver operating characteristic (ROC) curve confirmed their observation. However, in our evaluation of the ARK LZD kit, we observed more accurate results at higher concentrations with an acceptable upper limit of quantification at 18.5 mg/L. The good performances of the ARK kit in the analytical range validated was confirmed with our Bland-Altman comparison showing only one non-concordant result and a 95% limits of agreement thinner than those of Castoldi et al. The validated range of the ARK LZD assay was acceptable considering trough therapeutic targets of 2–8 mg/L [16]. On the other hand, for the TDM of tuberculosis patients, the recommendation is to measure the serum concentration 2 h post-administration (C_{max}) and possibly at 6 h in order to distinguish a delayed absorption from malabsorption [10,40]. In this particular context, the assay must display a wide analytical range because the normal C_{max} of LZD is 12–26 mg/L [10,41]. The UHPLC-PDA method, presenting a wider analytical range and a higher accuracy at high concentrations, is thus more adequate than the immunoassay. Moreover, as discussed above, for patients treated by a combination beta-lactams/LZD, the chromatographic method allowing their simultaneous quantification is an advantage. A solution investigated and validated by Castoldi et al. to overcome the limitation of the immunoassay range was to repeat the measurement after sample dilution. The turnaround time of results stay short but doubles the costs.

In conclusion, both assays showed suitable performances to perform routine TDM of linezolid. The choice of the method should be made according to the degree of emergency of the response required and the field of application justifying or not the simultaneous quantification of beta-lactams and LZD.

Credit author statement

D. Fage: Investigation; Methodology; Writing - original draft. G. Deprez: Investigation. B. Fontaine: Investigation. F. Wolff: Writing - review & editing. F. Cotton: Writing - review & editing; Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.121641.

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