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A method to determine two antibiotics prescribed to treat nosocomial infections in plasma and urine by micellar liquid chromatography

Juan Peris-Vicente [a,*], Jaume Albiol-Chiva [b], Devasish Bose [c], Abhilasha Durgbanshi [d], Samuel Carda-Broch [b]

[a] Department of Analytical Chemistry, Faculty of Chemistry, Universitat de València, 46100 Burjassot, Spain
[b] Department of Physical and Analytical Chemistry, ESTCE, Universitat Jaume I, 12071 Castelló, Spain
[c] Department of Criminology and Forensic Science, Doctor Harisingh Gour Vishwavidyalaya (A Central University), Sagar, Madhya Pradesh 470003, India
[d] Department of Chemistry, Doctor Harisingh Gour Vishwavidyalaya (A Central University), Sagar, Madhya Pradesh 470003, India

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ABSTRACT

Combined prescription of the antimicrobial drugs linezolid and meropenem is a common strategy to treat multidrug-resistant nosocomial infections. We propose an innovative method to determine these two drugs in plasma and urine, based on micellar liquid chromatography. Both biological fluids were diluted in mobile phase, filtered and directly injected, without any extraction step. Using a C18 column and a mobile phase of 0.1 M sodium dodecyl sulfate – 10 % methanol, phosphate buffered at pH 3, running under isocratic mode, both antibiotics were eluted without overlapping in<15 min. Detection was by absorbance: 255 nm for linezolid and 310 nm for meropenem. The influence of sodium dodecyl sulfate and methanol concentration on retention factor was established for both drugs using an interpretative approach assisted by chemometrics. The procedure was successfully validated following the guidelines of 2018 Bioanalytical Method Validation Guidance for Industry in terms of: linearity (determination coefficients over 0.99990), calibration range (1 – 50 mg/L), instrumental and method sensitivity, trueness (bias of −10.8 to + 2.4%), precision (relative standard deviation of < 10.2%), dilution integrity, carry-over effect, robustness and stability. It should be emphasized that the method uses low volumes of toxic and volatile solvents and can be achieved in a short period. The procedure was found useful for routine analysis, as it was cost-affordable, more eco-friendly and safer than hydroorganic HPLC, easy-to-handle and highly sample-throughput. Finally, it was applied to incurred samples of patients taking this medication.

1. Introduction

The administration of antibiotics has been restricted since people have abused them in the past. Bacteria multiply every 20 min and sometimes undergo mutations that are, by chance, a shield against an antibiotic. Moreover, they can pass these new resistance genes to other nearby bacteria, even from other families. The more antibiotics are used; the more bacteria evolve to resist them, and then the current antibiotic arsenal becomes obsolete, which poses in danger patient's health. An estimated 700,000 mortalities occur annually due to unsuccessful antibiotic treatments and that number is *in crescendo*. Now, the SARS-CoV-2 virus has made the silent pandemic of superbacteria worse. From February to March 2020, in Spain, the use of azithromycin increased by 400%, an antibiotic used desperately against the SARS-CoV-2 virus, doxycycline consumption increased 517% and the use of the main antimicrobial agents increased 100–200%, which may stimulate the increase to the development of resistant pathogens to these drugs. Thus, it is important to control the administration of antibiotics [1–3].

Patients in hospitals are susceptible to nosocomial infections, especially those in intensive care units. These are dangerous and difficult to manage, for many hospital pathogens are drug-resistant, the immune system of the patient is in a weak state, and this infection has to be treated simultaneously to the original illness [4]. In hospital-acquired infections, patients very often receive empiric broad-spectrum combination therapy for the first days of therapy until the infective pathogen is identified. A typical broad-spectrum combination for initial therapy comprises linezolid (LZD) combined with meropenem (MPN), as a significant number of these infections are caused by drug-resistant bacteria [5]. This combination has been found especially useful against infections caused by methicillin-resistant Staphylococcus aureus (MRSA)

* Corresponding author.
E-mail address: juan.peris@uv.es (J. Peris-Vicente).

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[6] and methicillin-susceptible Staphylococcus aureus (MSSA) [7]. Meropenem is a carbapenem with antibacterial activity widely used against a wide range of Gram-positive and Gram-negative bacteria [8]. Linezolid is an oxazolidinone derivative that has a predominantly bacteriostatic effect against severe infections caused by methicillin or vancomycin resistant Gram-positive bacteria [9]. The structure of these compounds can be seen in [9], while the physicochemical properties are in Table 1 [10]. Also, a combination of both compounds is often administered to treat endophthalmitis [11] and multi- and extensively drug-resistant tuberculosis [12].

Therapeutic drug monitoring (TDM) is recommended for patients receiving linezolid/meropenem combinations [13,14]. Indeed, there is a large inter-individual variability of pharmacokinetics for a same recommended posology during the duration of the treatment, caused by drug-drug interactions and the alteration of the metabolism caused by the disease. It can be useful to verify the concentration of the drugs remains over the minimum inhibitory concentration, while avoiding overexposure, and adjust the dose accordingly. This could avoid the generation of drug-resistant bacteria and the side effects, along with a significant improvement of the clinical success of the treatment. Therefore, clinicians require analytical methods to determine these drugs in plasma and urine [15–18].

These antibiotics have been determined alone or in combination with others, by high performance liquid chromatography (HPLC) assays with ultraviolet (UV) detection [5,15,19–21]. These assays have some disadvantages in terms of extensive chromatographic run times, a large consumption of non-environmentally friendly solvents and long sample treatments. Also, HPLC methods coupled with mass spectrometry (LC–MS/MS) for determination of the selected drugs in human plasma have also been published [13,14,16,17,22–30]. Nowadays, more and more scientists are focusing on exploring the application of MS due to its excellent selectivity and sensitivity. However, the high cost involved in the instrumental setup and maintenance makes it unaffordable for most laboratories.

Micellar liquid chromatography (MLC), using sodium dodecyl sulfate (SDS) as a surfactant, allows biological samples, like plasma and urine, to be analyzed without needing to eliminate proteins and other interfering substances, thus considerably reducing the cost and analysis time. In MLC, the retention behavior of compounds can be predicted with high accuracy. This fact simplifies the optimization of the mobile phase composition [18]. In addition, one of the main applications of MLC is direct sample injection of biological material into the column, due to the ability of micellar aggregates to dissolve sample proteins and other compounds. MLC technique has proved to be a useful technique in the determination of diverse groups of drugs in serum and urine samples [31–33].

Table 1
Physico-chemical parameters [10], Linearity and sensitivity parameters for both analytes (SD = standard deviation, concentrations in mg/L).

	Linezolid	Meropenem
Solubility in water	>1000 mg/L	>1000 mg/L
Log Po/w (hydrophobicity)	0.64	−0.69
Acidic/alkaline activity	None	pKa(COOH) = 3.5 pKa(secondary amine protonated) = 9.4
Formal charge at pH 3/5/7	0/0/0	+0.76/+0.03/+0
Slope ± SD	60.99 ± 0.09	17.94 ± 0.08
y-intercept ± SD	0.5 ± 0.4	−0.1 ± 0.4
r^2	0.99998	0.99990
RRSD (%)	0.5	1.1
Instrumental LOD	0.02	0.08
Instrumental LLOQ	0.05	0.20
MDL	0.1	0.4
MQL	0.25	1.0

The objective of the research was the proposal of a method to determine the antibiotics linezolid and meropenem in plasma and urine for clinical purposes using micellar liquid chromatography. It should be reliable, rapid, economic, practical and have a high sample throughput. It should be greener than hydroorganic RP-HPLC. Its analytical performances will be evaluated following the guidelines of the Food and Drug Administration (FDA) 2018 Bioanalytical Method Validation Guidance for Industry [34] and the fundamentals of retention mechanism will be investigated, in order to find out the effect of the composition of the mobile phase on the retention. Finally, the procedure was used to analyze patient samples to determine its appropriateness for routine analysis.

2. Material and methods

2.1. Reagents, chemicals and apparatus

Powdered standards of linezolid and meropenem trihidrate, both purity > 98% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (>99.0%) and sodium dihydrogen phosphate dihydrate (>99%) were supplied by Scharlab (Barcelona, Spain). Hydrochloric acid (nearly 37%) and sodium hydroxide (>98%) were come from Panreac (Barcelona). Methanol (HPLC grade) was bought from J.T. Baker (Deventer, Holland). Ultrapure water was made at the laboratory using a generator device Simplicity UV (Millipore S.A.S., Molsheim, France) from running deionized water provided by the University. This water was used to prepare all the aqueous solutions.

An analytical balance Metter-Toledo (Greifensee, Switzerland) was used to weigh the solid standard. pH measurements were performed using a GLP 22 potentiometer equipped with a combined Ag/AgCl/glass electrode (Crison, Barcelona, Spain) daily calibrated. An ultrasonic bath Ultrasons-H (Selecta, Abrera, Spain) was used to ultrasonicate the solutions.

Stock solutions of linezolid and meropenem (100 mg/L) were prepared in ultrapure water and ultrasonicated. Working solutions were made by successive dilutions in ultrapure water. All the solutions were kept in amber vials at + 4 °C for one month. Preparation of micellar solutions is described in [33].

2.2. Chromatographic conditions

An HP1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, a degasser, an autosampler with a 20-μL loop and a photodiode array detector; connected to a PC. The software HP ChemStation Rev. B.01.01 (Agilent Technologies) was employed to control the instrumentation, as well as to visualize, process and store the signals. The column was a Kromasil C18 (Scharlab, Barcelona) with the following characteristics: length, 15 cm; internal diameter, 4.6 mm; particle size, 5 μm; pore size, 10 nm). The mobile phase was an aqueous solution of SDS 0.1 M – 10% methanol – 0.01 M phosphate buffer at pH 3; running at 1 mL/min under isocratic mode at room temperature. The column pressure was nearly 150 bars and remained quite invariant throughout all the analyses. Absorbance detection wavelength was 255 nm from 0 to 7.9 min, and 310 nm from 8.0 to 15 min. To-be-injected solutions were filtered through a 0.45 μm membrane filter by hand pushing using a 3-mL syringe and introduced in chromatographic vials. Non injected parts were discarded.

The specific recommendations for HPLC-instrumentation working and cleaning when utilizing micellar mobile phases are detailed in [35]. The dead time (t_0 = 1.0 min), as the time were the signal starts to rise because of the front, and the retention time (t_R, min) and peak width (interpolated w and at 5%-height $w_{0.05}$ min) of each solute were directly taken from the chromatograph. The retention factor (k), efficiency (number of theoretical plates), asymmetry factor and resolution were calculated as in [36].

2.3. Sample collection and treatment

Incurred plasma and urine samples were obtained from patients suffering some infectious diseases and following a therapy based on linezolid and/or meropenem. Blank samples were from three healthy volunteers taking no-medication. All samples were extracted by qualified medical staff and provided by a local Hospital. The investigation was approved by two local Ethics Committees, Hospital and the University Ethic Committee for Analysis of Research Projects. Written informed consent was obtained from all participants and all research was performed in accordance with the 2013 Helsinki Declaration principles.

Samples were sent unlabeled and neither clinical nor personal information was provided to the laboratory for confidentiality reasons. The laboratory committed to destroy all samples from patients, and all chromatograms and experimental data (except that published), immediately after the publication of the paper. Samples from patients were only analyzed as part of this specific study. The laboratory agreed not to transmit any information to other institutions.

Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK), and were centrifuged for 5 min at 756 Relative Centrifugal Force or G-force at 4 °C, to get the non-cellular fraction. Plasma was immediately frozen and stored at − 20 °C. Urine samples were collected in glass tubes and kept at the same temperature. Samples of biological fluids, either blank or patient samples, were treated the same way. On the day of analysis, they were thawed at room temperature. Afterwards, an aliquot was 1/5-dissolved in mobile phase, filtered, and directly injected. The spiking was performed by adding the appropriate volume of a standard solution of the analytes, before dilution. All processed but unused samples were discarded.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The combined use of a 15-cm-C18 column and hybrid mobile phases of sodium dodecyl sulfate running under isocratic mode at 1 mL/min has been proven as a valuable approach to determine cationic and neutral drugs in biological fluids (plasma and urine) [18]. In the frame of this research, we optimized the specific chromatographic conditions to maximize resolution and sensitivity and minimize analysis time: concentration of SDS ([SDS]), type and proportion ([alcohol], φ %, v/v) of organic solvent, pH (restrained to the working interval of alkyl-silica based columns, 2.5–7.5) and detection conditions. Experiments were performed using a solution containing 1 mg/L of both compounds.

Both drugs are quite polar, and the contribution of hydrophobic interaction in the retention was quite limited (Table 1) [10]. Moderate retention was expected, and would be dependent on electrostatics. Linezolid keeps its formal charge + 0 in the entire interval and the retention factor is nearly independent form pH. Meropenem displays formal charge of + 0 at 7.0; +0.03 at and 5.0; and a formal charge of + 0.76 at pH 3; it was therefore expected that its retention would increase at pH 3 (Table 1). Hence, we selected this value as optimal, in order to increase the retention of meropenem.

Short-chained monoalcohols are commonly added to micellar mobile phases to increase the efficiency and elution power. Their effect on these parameters increases with their molecular mass. Alcohols more hydrophobic than 1-propanol were discarded because the analytes would elute at the dead time. Therefore, methanol was finally selected, as it ameliorates peak shape while not excessively affecting retention [37].

The [SDS] and φ were simultaneously optimized by studying their effect on the retention, following an interpretative strategy, in order to evaluate their possible interaction and to limit the number of assays. We used a 3^{k} factorial design with k = 2 [38], and the values were the minimum, central and maximum values, 0.05–0.15 M for SDS and 5–15% of methanol. Furthermore, a mathematical model was constructed to predict the retention factor; and indirectly the resolution and analysis time; from the composition of the mobile phase. For that, the experimental data ([SDS] and [methanol] normalized between the interval −1 to + 1 in order to better compare the constants regardless of the magnitude each factor) were used to adjust the equation indicated in Table 2, which has been proven to be useful to model retention for polar to moderately hydrophobic drugs in MLC [18], by curve-fitting non-linear least-square regression [39]. The parameter c_0 indicates the inverse of the retention factor at average concentrations, while the effect of the concentration of SDS, the proportion of methanol and their interaction are quantified by c_1, c_2 and c_{12} respectively. The tested mobile phases and results can be seen in Table 2.

The model exhibits acceptable goodness-for-fit, according to the values for the multiple coefficients of determination (R^2). Therefore, the equation can be used to monitor the retention time at intermediate values. Retention for meropenem was higher than for linezolid, for the effect of electrostatic interactions between the positively charged solute and the negative layer on the stationary phase. Otherwise, the effect of the factors, excluding the interaction, was similar for both compounds.

Meropenem was retained more than linezolid, despite its lower hydrophobicity, because it is cationic and interacts by electrostatics with the anionic sulfate groups of SDS-monomers adsorbed on the mobile phase and oriented towards the mobile phase. We determined that the retention diminishes at increasing values of [SDS], thus pointing to a binding behavior between the analytes and the micelles in the mobile phase. This interaction was mainly by electrostatics for meropenem. The elution power of the mobile phase increases with the proportion of methanol, as expected. For linezolid, the interaction was positive, yet not very significant, suggesting the effect of methanol is slightly increased at high concentrations of SDS, and *viceversa*. At a higher proportion of methanol, the aggregation number of SDS augments, but this effect is compensated for by the increase of the critical micellar concentration, and then the number of micelles barely changes [40]. However, the interaction was positive, but not significant, for meropenem. Finally, all the parameters were included in the model.

The optimal combination of SDS and methanol concentration in the mobile phase was selected following the criteria: maximum difference in retention time ($\Delta t = t_R$Meropenem – t_RLinezolid) – minimum analysis time (t_RMeropenem + 3 min, to guarantee its complete elution and the return of the signal to the baseline level). These were predicted using the equations adjusted for the retention factor. The visualization of Δt v.s. [SDS] and [methanol] can be seen in the Fig. 1.

We found that at increasing values of [SDS] and [methanol], both Δt

Table 2
Experimental data of retention and adjusted equation for both drugs (freedom degree = 5).

[SDS] (mol/L)	[methanol] (% v/v)	*k*(linezolid)	*k*(meropenem)
0.05	5	9.934	19.494
0.05	10	4.464	10.425
0.05	15	4.346	5.346
0.1	5	3.967	8.143
0.1	10	3.838	7.779
0.1	15	2.333	4.649
0.15	5	3.759	6.114
0.15	10	1.978	3.893
0.15	15	1.684	3.15
Equation: $1/k = c_0 + c_1$ [SDS] + c_2 [methanol] + c_{12} [SDS][methanol]			
		Linezolid	Meropenem
$c_0 \pm SD_{c0}$ (p_0)		0.318 ± 0.016 (<0.000)	0.171 ± 0.009 (<0.000)
$c_1 \pm SD_{c1}$ (p_1)		0.135 ± 0.019 (0.001)	0.067 ± 0.011 (0.002)
$c_2 \pm SD_{c2}$ (p_2)		0.106 ± 0.019 (0.003)	0.064 ± 0.011 (0.002)
$c_{12} \pm SDc_{12}$ (p_{12})		0.05 ± 0.02 (0.09)	0.005 ± 0.013 (0.7)
R^2		0.94	0.93

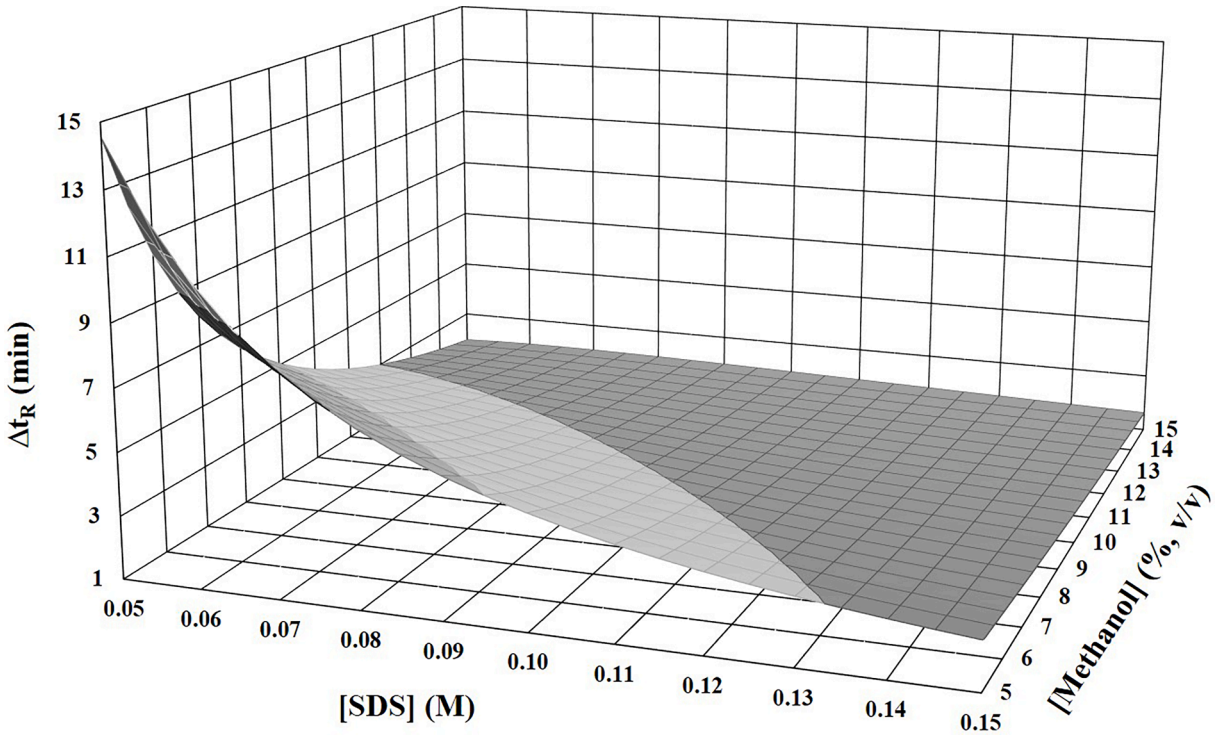

Fig. 1. Three-dimensional response surface of difference in retention time between meropenem and linezolid (Δt_R) when varying [SDS] and methanol proportion.

and analysis time decreased. Mobile phases providing Δt_R under 2.5 min and t_RMeropenem > 10 min were directly discarded. Finally, the optimal conditions were [SDS] = 0.1 M and [methanol] = 10%.

The detection wavelength was optimized to maximize the signal-to-noise ratio. Both drugs were determined using the previously selected separation conditions, and the UV–Visible absorption spectra was in-time registered (Fig. 2). Maximum absorbance was 255 nm for linezolid and 310 nm for meropenem. In order to determine each drug at their maximal signal-to-noise ratio, the registered signal was the absorbance at 255 nm from the start of the chromatogram to the end of linezolid elution (0 to 7.9 min) and then shifted to 310 nm before the elution of meropenem to the end of run (8.0 to 15 min).

In order to state the reliability of the experimental conditions, a system suitability testing was performed by replicate injections of a solution containing 2 mg/L for both drugs under the optimized conditions (n = 5). These were valid, as the parameters calculated from the chromatographic responses comply with the acceptance criteria stated by FDA (Table 3) [36]. Otherwise, UV–Visible absorption spectra were taken at the retention time, 50%- and 5%-leading and tailing edges for peak purity studies.

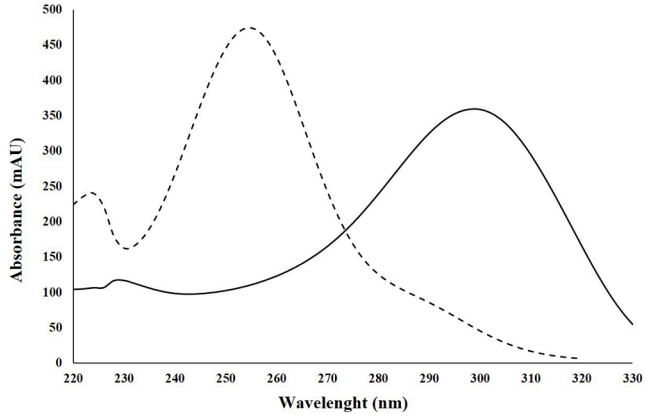

Fig. 2. Absorbance spectrum for linezolid (– – – – – – –) and meropenem (———) obtained in time during the chromatographic run at the maximum retention time using the optimal experimental conditions.

3.2. Method Validation

The method was validated following the guidelines of the FDA 2018 Bioanalytical Method Validation Guidance for Industry, which is specifically devoted to the determination of drugs in biological fluids [34], and with the assistance of other relevant publications on validation [41,42]. The studied validation parameters were calibration range, linearity, instrumental sensitivity, robustness (in stock solution), selectivity, method sensitivity, trueness, precision, dilution integrity, carry-over effect (in blank samples) and stability.

3.2.1. Calibration range, linearity and instrumental sensitivity

Although FDA guidelines require the calibration standards in the same biological matrix as the samples in the intended study, it has been previously demonstrated the absence of matrix effects when analyzing plasma or urine by MLC [35]. Therefore, the calibration was performed in standard solution prepared in mobile phase, to facilitate the simultaneous analysis of both biological fluids using the same calibration solutions in routine analysis.

Several solutions containing increasing concentrations of linezolid and meropenem (up to 10 mg/L) were prepared in mobile phase and injected in triplicate. The data series was found to be homoscedastic, as

Table 3
Results of the system suitability test.

Parameter	Linezolid	Meropenem	Acceptance criteria
t_R (min) (RSD, %)	4.88 ± 0.04 (0.8)	8.82 ± 0.08 (0.8)	RSD ≤ 1%
RSD of peak area	0.9	1.0	≤1%
retention factor	4.9	8.8	≥2.0
Efficiency	2052	2328	≥2000
Asymmetry factor	1.8	1.2	≤2
t_0 = 1.0 min			

the obtained variances for each concentration were significantly similar by a F-Snedecor test at 5% significance level. The average peak area was plotted *v.s.* the concentration by least-square linear regression, to calculate the slope, y-intercept, the determination coefficient (r^2) and the relative residual standard deviation (RRSD), by linear least-square regression. The instrumental lower limit of quantification (LLOQ) was the lower concentration wherein the predicted and experimental values were similar. The instrumental limit of detection (LOD) was set to 3.3 times the deviation standard of the blank (estimated at the standard deviation of the y-intercept), divided by the sensitivity factor (estimated by the slope of the calibration curve). The upper limit of quantification (ULOQ) was 10 mg/L. The results can be seen in Table 1.

For each drug, adequate linearity was proven inside the corresponding linear range, according to the plot residual *v.s.* concentration (residual were normally distributed around 0 and no trend was visualized), determination coefficients (r^2) were > 0.995, RRSD values were < 1 and all the calibrators were $< 15\%$ the nominal values. No outliers were detected, as the relative residual were > 3 and the Cook squared distance was < 1. Y-intercept confidence interval included 0, at a significant level of 5%, and then the curve does not exhibit a systematic error.

3.2.2. Selectivity

This parameter was evaluated independently for plasma and urine. In both cases, a blank sample was analyzed. The obtained chromatograms are shown in Fig. 3A for plasma and 3B for urine. We observed a broad band and several minor peaks, corresponding to some endogenous compounds of the matrix, from the dead time to nearly 3 min; and a quite stable baseline at higher elution times. No peaks were noticed at the window time of the analytes.

The same blank sample was spiked at 5 mg/L (to imitate a sample containing 5 mg/L before being diluted) of both compounds, and reanalyzed. Chromatograms can be seen in Fig. 3C for spiked plasma and 3D for fortified urine. The chromatogram background was like the blank, and no other matrix peaks were detected. Linezolid and meropenem peaks were resolved enough to avoid overlapping between them or with the matrix band. These were also compared to those obtained by the analysis of the standard solutions (section 3.1) overlapping and exhibited similar shape.

A peak purity study was performed by taking the UV-absorbance spectra of the analytes in the fortified sample at the retention time, and 50%- and 5%- height of leading and tailing edge. These were compared to that obtained from the analysis of the standard solution, and were found to be similar, thus pointing out the absence of coeluting compounds.

From this study, we deduce the peaks can be reliably identified as the analytes from the retention time, and the entire corresponding peak area can be assigned to them in the quantification.

3.2.3. Method detection and quantification limits

The method detection and quantification limits (MDL and MQL, respectively) were calculated from the instrumental ones, considering the dilution factor applied in the sample treatment (1/5). The results are shown in Table 1. The method upper quantification limit (MUQL) was calculated the same way from the ULOQ; and was 50 mg/L.

The response of the zero calibrator in sample (blank in 3.2.2) was less than five times that obtained by a blank sample spiked at the MQL. Otherwise, results obtained in trueness and precision at this level were $< 20\%$. Therefore, the method can detect the drug at the concentrations they may be found in plasma and urine samples of patients taking this medication.

3.2.4. Trueness, precision and dilution integrity

These parameters were determined, within-run and between runs using quality control (QC) samples of plasma and urine at four levels (LLOQ, low, medium and high), which were prepared by spiking blank

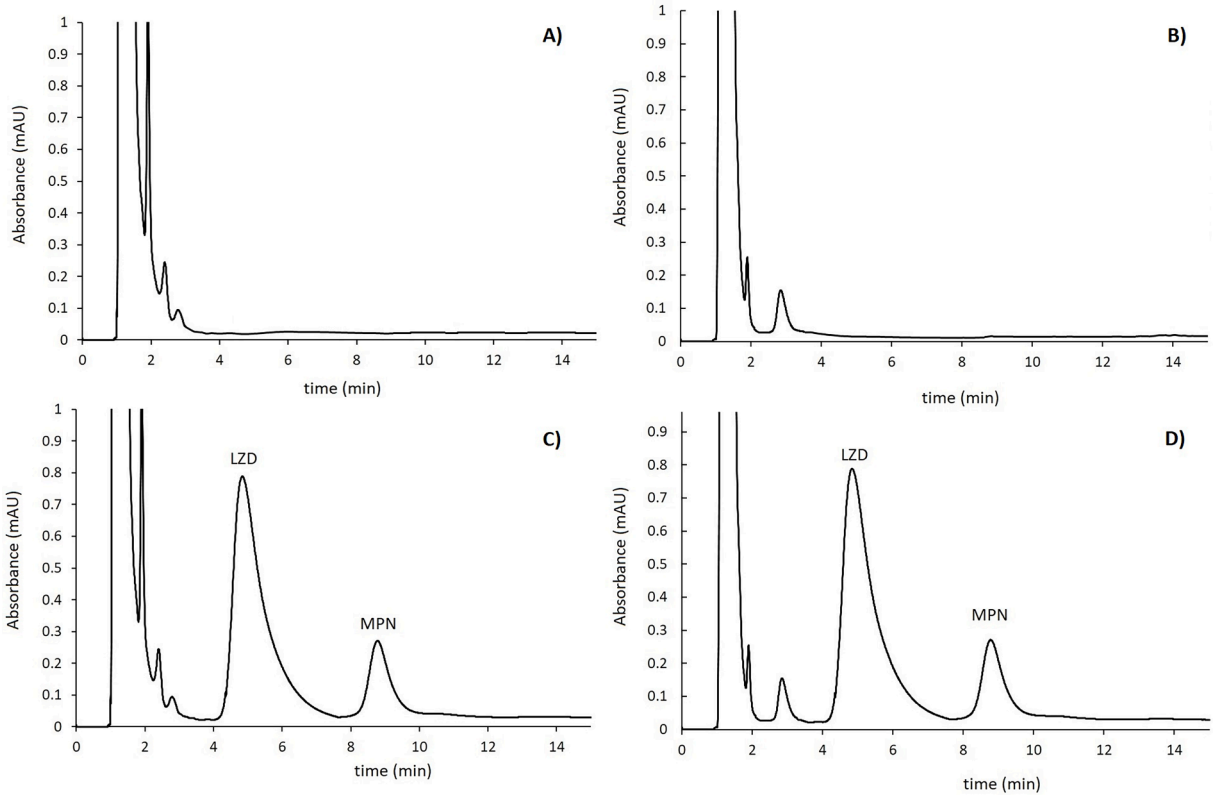

Fig. 3. Chromatogram obtained by the analysis under the optimized conditions of a A) blank plasma; B) blank urine; C) blank plasma fortified with 5 mg/L of linezolid (LZD) and meropenem (MPN) and D) blank urine spiked at 5 mg/L of both analytes (the concentration refers to the sample before being diluted).

samples at the corresponding concentration. A fifth level, over the UMQL was added to evaluate dilution integrity. In this case, an extra-1/5-dilution in mobile phase was performed to adjust the drug concentrations of the injected solution to the linear interval.

For within-run studies, six samples (by each level and matrix) were successively analyzed using the following procedure. Trueness was calculated as bias, by the difference between the found concentration and the nominal value, divided by the nominal value, while the precision was quantified by the relative standard deviation of the six found concentrations. Between-run parameters were determined by repeating the above-described approach five times (using renewed solutions and calibration curves) over a 3-month period. Between-run trueness was the difference between the grand mean (average of all the found concentrations) and the nominal value, divided by the nominal value, while between-run precision was the relative standard deviation of all the found concentrations. Results can be seen in Table 4.

Values for bias (-10.8 to + 2.4%) and dispersion (<10.2%) were inside the requirements of the guideline (<15%), thus indicating the procedure provides reliable quantitative results. Besides, strongly concentrated samples can be analyzed, after the proper dilution. This was attained by the simplicity of the pretreatment, thanks to the strong solubilization ability of micellar solution, as the sample is quantitatively introduced in the column and extraction/purification steps are avoided.

3.2.5. Carry-over effect

This parameter was examined independently for urine and plasma. In order to evaluate this parameter, a blank sample fortified at 50 mg/L of both drugs and a blank sample were processed and injected consecutively (chromatographic run of 15 min). No peak was observed in the chromatogram corresponding to the blank sample at the window time of the analyzed. Therefore, samples with drug level up to 50 mg/L can be analyzed in the same sequence run without risk of cross contamination.

3.2.6. Robustness

We evaluate the alteration of the main chromatographic responses (i.e., retention time and peak area) caused by small fluctuation of instrumental conditions deliberately introduced, by using a Youden approach with 6 factors [43]. This kind of strategy is stronger than a one-factor-a-time analysis, as it allows the evaluation of each factor when the other ones are also outside the optimal value. The assayed interval of oscillations from the optimal value were those that may occur during the usual work in the laboratory. The studied factor and their corresponding upper/lower levels were: A) UV-absorbance wavelength, λabs ± 5 nm; B) concentration of SDS, 0.105–0.095 M; C) methanol proportion in mobile phase, 10.5/9.5%, D) pH of the mobile phase, 3.2/2.8; E) flow rate, 1.05/0.95 mL/min and F) injection volume, 22/18 μL. A standard solution containing 1 mg/L of both drugs was analyzed by eight different sets of experimental conditions, wherein the level of each factor was fixed by the experimental design. The effect of each factor was quantified by the difference of the average response obtained at the upper limit and the lower limit, divided by the average value of the eight measurements. Relative differences > 5% were considered significant.

The increase of the flow rate provokes a noticeable decrease of retention time and peak area for both drugs. Otherwise, for both drugs the peak area substantially augments with the injection volume, which was expected, as the amount of drug is higher. What's more, SDS concentration exhibited a significant influence on retention time for linezolid. For meropenem, detection wavelength affected the peak area, and the increase of mobile phase pH strongly reduced the retention time. As the studied pHs are close to the pKa of its carboxylic group (3.5), there is a significant shift of the formal charge to less positive values, thus reducing its interaction with the sulfate groups on the stationary phase. Finally, in order to maintain the quality of the results, attention must be paid to these parameters when performing the analyses.

Table 4
Within-run and between-run trueness and precision ([a]n = 6; [b]n = 5) for linezolid and meropemem. Acceptance criteria: <20% for MQL and < 15% at higher levels.

	Linezolid			
	Plasma		Urine	
Concentration	Within-run[a] Trueness/ Precision	Between-run[b] Trueness/ Precision	Within-run[a] Trueness/ Precision	Between-run[b] Trueness/ Precision
0.25	−10.8/8.9	−9.3/10.2	−9.3/8.4	−8.5/9.7
5	1.8/3.0	2.0/3.9	1.5/3.3	2.3/4.0
20	−1.3/1.4	−1.1/3.3	−2.0/2.9	−1.9/3.1
35	−1.4/1.3	−0.9/2.6	+1.3/4.3	+0.8/5.0
75 (1/5 dil.)	−2.3/1.4	−2.0/2.5	−0.9/2.1	−0.5/2.8
	Meropenem			
Concentration	Within-run[a] Trueness/ Precision	Between-run[b] Trueness/ Precision	Within-run[a] Trueness/ Precision	Between-run[b] Trueness/ Precision
1.0	−8.7/7.6	−8.0/8.3	−7.2/9.0	−6.8/9.4
5	−5.1/1.2	−4.3/2.6	−5.3/3.6	−4.7/4.0
20	+1.2/0.4	+0.9/1.3	+1.1/2.4	+0.8/3.1
35	+0.5/0.8	+0.7/1.9	+0.3/2.0	+0.5/2.8
75 (1/5 dil.)	+2.4/0.6	+1.3/2.6	+1.9/3.0	+1.5/2.4

3.2.7. Stability

The resistance of linezolid to chemical changes through time was studied in different environments (stock solution, benchtop/autosampler, freeze–thaw and long-term) at the usual storage conditions by monitoring the decrease of peak area, compared to the fresh sample (analysis of the day zero). The decay was considered significant if > 5% for stock solutions and > 15% for the other cases. Maximum storage time was the period required to reach a significant decomposition. Experiments were programmed as follows (in all cases a non-stored sample was analyzed, which was considered as fresh sample):

a) stock solution (at + 4 °C): a stock solution containing 1 mg/L of both drugs was prepared and kept in the fridge. Each two days, for two months. the solution was thawed, an aliquot analyzed, and replaced in the fridge.
b) benchtop/autosampler (room temperature): a blank sample fortified at 5 mg/L of both antibiotics was processed and placed in the autosampler tray. Each 30 min for one day, an aliquot was injected. The first injection was taken as the fresh sample.
c) freeze–thaw stability (at −20 °C): a blank sample was spiked at 5 mg/L of both drugs and stored in the freezer. Each three days, for a two-week period, the sample was thawed, an aliquot analyzed and the remaining solution replaced in the fridge.
d) long-term stability (at −20 °C): a set of blank plasma samples were fortified at 5 mg/L of both antibiotics and placed in the freezer. Each three days for 15 days, one sample was thawed, analyzed and then discarded.

Tests b), c) and d) were separately performed for urine and plasma.

No significant degradation was noticed in any of the situations for both antibiotics, and then the storage time was that of the duration of each assay. Therefore, the stock solutions were kept for two months, and then renewed. The processed sample can be analyzed up to one day after dilution in mobile phase. Therefore, when many samples have to be analyzed in a workday, the entire set can be diluted and then placed in the autosampler and successively injected. Besides, many samples can be analyzed the same day. Otherwise, plasma and urine samples can be kept in a freezer for 15 days before analysis and can withstand freeze–thaw cycles during this period.

3.3. Analysis of incurred samples

The procedure was employed to the analysis of plasma (P) and urine (U) samples from patients suffering from multidrug-resistant nosocomial

infections, and following a therapy based on the combined prescription of linezolid and meropenem. The quality of the results was checked by analyzing quality control (6 QC), samples blanks and incurred sample reanalysis (ISR) in the same run. All the samples were simultaneously processed and then put in the autosampler for injection. Results are shown in Table 5 (P and U samples are not correlated). Fig. 4A and 4B depict the chromatogram obtained by the analysis of samples P2 and U2, respectively.

Results obtained by the samples introduced to appraise the quality of the response were inside their respective acceptance criteria (QC < 15% bias from the nominal value, blank < 20% of LLOQ signal and ISR < 20% bias from the average value), and thus the quantitative results of the entire run can be considered as reliable. Moreover, both antimicrobial drugs were resolved from the matrix.

Only economic and widely available material, reagents and instrumentation were used. In spite of the large number of samples or solutions (32), the entire set was analyzed in a single day, thus the procedure exhibits a high sample throughput. This performance was accomplished by the strong solubilizing power of micellar solutions. Consequently, the cost of the analysis was limited. Besides, the procedure is more environmentally friendly and safer than typical RP-HPLC procedures, as the quantity of hazardous chemicals handled and wasted was minimal (only 10% of volatile and toxic organic solvent in the solutions used in the pretreatment as in mobile phase)..

4. Conclusions

The analysis of plasma and urine to quantify the antibiotics linezolid and meropenem is feasible by micellar liquid chromatography. The main feature of the procedure is the simplification of the sample treatment, which is limited to a direct injection of the diluted sample, thus avoiding cumbersome and time-consuming extraction steps. The analytes were resolved from the matrix in<12 min. Acceptable analytical performances were obtained, as the method was satisfactorily validated by the guidelines of the FDA 2018 Bioanalytical Method Validation Guidance for Industry. Results of trueness and precision were similar to that provided by gold-standard methods. Linear range were sufficient to detect the antimicrobial drugs at their usual concentrations in plasma and urine. The main limitation was the lower sensitivity and selectivity, if compared to LC-MS, and the use of salty mobile phases, which forced to implement a rigorous cleaning protocol. To sum up, the procedure was found to be reliable, widely available, relatively sustainable, which makes it useful for routine analysis in a clinical laboratory. Besides, the effect of SDS and methanol concentration in the mobile phase on retention was established for both drugs.

Data availability statement

The data are not publicly available because they contain information that could compromise research participants.

CRediT authorship contribution statement

Juan Peris-Vicente: Visualization, Conceptualization, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Jaume Albiol-Chiva:** Visualization, Writing – review & editing, Data curation, Investigation, Formal analysis, Software, Methodology. **Devasish Bose:** Project administration, Funding acquisition, Resources, Methodology. **Abhilasha Durgbanshi:** Supervision, Investigation, Conceptualization. **Samuel Carda-Broch:** Supervision, Methodology, Resources, Writing – original draft, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Juan Peris-Vicente, Jaume Albiol-Chiva, Devasish Bose, Abhilasha Durgbanshi and Samuel Carda-Broch declare that they have no conflicts of interest that might be relevant to the contents of this manuscript. The authors alone are responsible for the content and writing of this article.].

Table 5
Analysis of incurred samples (concentrations in mg/L).

Sample	Linezolid	Meropenem	Sample	Linezolid	Meropenem
QC P 5 mg/L	4.8	5.1	QC U 5 mg/L	5.3	4.8
P1	18.6	17.3	U1	38.5	15.6
P2	10.7	28.6	U2	25.3	15.3
P3	8.7	4.9	U3	19.5	23.6
P4	0.85	2.6	U4	28.6	15.6
QC P 20 mg/L	18.7	19.3	QC U 20 mg/L	21.9	19.6
P5	13.6	31.3	U5	n.d.	2.8
P6	0.30	1.8	U6	43.6	32.4
P7	20.9	46.7	U7	16.7	22.5
P8	6.8	39.2	U8	32.2	26.7
QC P 35 mg/L	36.3	35.9	QC U 35 mg/L	33.8	34.2
P9	14.8	13.9	U9	23.6	17.9
P10	n.d.	23.5	U2-bis	18.7	24.2
P5-bis	14.1	32.56	U10	43.2	30.5
P11	7.8	4.6	U11	16.3	22.8
QC P Blank	n.d.	n.d.	QC U blank	n.d.	n.d.

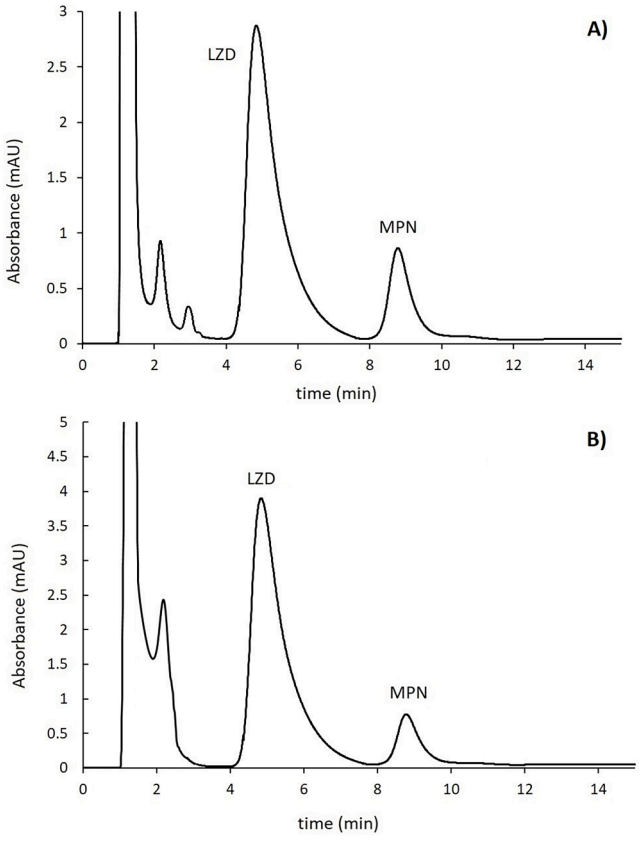

Fig. 4. Chromatogram obtained by the analysis of sample A) P2 and B) U2.

Data availability

The data that has been used is confidential.

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